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Hygiene management in farm animal housing

Assessment of hygiene indicators and critical points in sanitation

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Summary

Hygiene management in farm animal housing:
Assessment of hygiene indicators and critical points in sanitation

Cleaning and disinfection together form an important aspect of hygiene management in livestock farming. The professional implementation of these hygiene measures can help to interrupt infection chains, prevent the spread of diseases and antibiotic-resistant bacteria and maintain a high level of animal health, animal welfare and performance. Objective hygiene indicators are needed to evaluate the success of cleaning and disinfection. Therefore, an aim of this thesis was to test different hygiene indicators from the health and food sector with regard to their suitability for use in livestock production. The focus was on visual evaluation, two different rapid tests for the measurement of protein and adenosine triphosphate (ATP) residues, microbiological contact plates, microbiological swab samples, and microbiological sock samples for the evaluation of the total aerobic count and selected target organisms. The different hygiene indicators were tested in pig fattening farms and in newborn calf rearing facilities. The evaluation showed positive correlations between the individual methods. A visual check should always be carried out prior to disinfection, independent of the use of other indicator systems. The two rapid test systems can be used for in-house training, for the self-control of commercial cleaning companies or for audits. The swab samples are superior to the contact tests for the determination of the microbiological status in livestock farming. Microbiological tests are essential for assessing the presence of specific pathogens and cannot be replaced by rapid tests. To improve hygiene management, critical points were evaluated following cleaning and disinfection in pig fattening farms, calf housing facilities and chicken fattening farms. Especially the drinking and feeding equipment was often found to be insufficiently cleaned and highly contaminated, with an average value for the total microbial count of 5.6 $\log_{10}$ cfu · cm^{-2} in drinkers in fattening pig houses and 5.3 $\log_{10}$ cfu · cm^{-2} in feeding troughs in fattening pig houses as well as in milk feeding buckets and artificial teats for suckling calves. After cleaning and disinfection, *Pseudomonas aeruginosa* was detected in the water pipes of the chicken fattening farm at all sampling points, additionally, most of them showed resistance to antibiotics. Monitoring the cleaning efficiency and careful training of staff can help to improve hygiene and reduce the spread of pathogenic and antibiotic-resistant bacteria. Special attention should be paid to critical points such as feeding and drinking equipment and pipes.

Zusammenfassung

Hygienemanagement in der Nutztierhaltung:
Bewertung von Hygieneindikatoren und kritischen Punkten

Reinigung und Desinfektion bilden zusammen eine wichtige Säule des Hygienemanagements in der Nutztierhaltung. Die fachgerechte Durchführung dieser Hygienemaßnahmen kann dabei helfen, Infektionsketten zu durchbrechen, die Ausbreitung von Krankheiten und antibiotikaresistenten Keimen zu verhindern und so eine stabile Tiergesundheit und damit auch Wohlergehen und Leistung zu ermöglichen. Für die Bewertung des Reinigungs- und Desinfektionserfolgs sind objektive Hygieneindikatoren erforderlich. Daher war es ein Ziel dieser Arbeit, verschiedene Indikatoren aus dem Gesundheits- und Lebensmittelbereich hinsichtlich ihrer Eignung zur Bewertung des Hygienestatus in der Nutztierhaltung zu testen. Im Fokus standen dabei die visuelle Begutachtung, zwei Schnelltests zur Messung von Protein- und Adenosintriphosphat-(ATP)-Rückständen, mikrobiologische Abklatschtests sowie mikrobiologische Tupfer und Sockenproben zur Bewertung der aeroben Gesamtkeimzahl und zur Detektion ausgewählter Zielorganismen. Die verschiedenen Hygieneindikatoren wurden in Schweinemastbetrieben und in der Saugkälberhaltung getestet. Bei der Auswertung zeigten sich positive Korrelationen zwischen den einzelnen Methoden. Eine visuelle Kontrolle sollte immer vor der Desinfektion erfolgen, unabhängig vom Einsatz weiterer Indikatorsysteme. Bei den beiden Schnelltestsystemen ist ein Einsatz für innerbetriebliche Schulungen, für die Selbstkontrolle von gewerblichen Reinigungsfirmen oder für Audits vorstellbar. Die Tupferproben sind zur Erfassung des mikrobiologischen Status in der Nutztierhaltung den Abklatschtests überlegen. Mikrobiologische Untersuchungen sind für die Bewertung des Vorkommens spezifischer Erreger unabdingbar und können nicht durch Schnelltests ersetzt werden. Zur Verbesserung des Hygienemanagements wurden kritische Punkte im Anschluss an die erfolgte Reinigung und Desinfektion in der Schweinemast, der Kälbereinzelhaltung und in der Hähnchenmast evaluiert. Hierbei zeigten sich besonders die Tränke- und Fütterungseinrichtungen häufig als unzureichend gereinigt und mikrobiell hoch belastet, mit einem durchschnittlichen Wert für die Gesamtkeimzahl von 5,6 $\log_{10}$ cfu $\cdot$ cm^{-2} in Tränken in Mastschweineställen und 5,3 $\log_{10}$ cfu $\cdot$ cm^{-2} in Trögen in Mastschweineställen sowie in Milchtränkeeimern für Saugkälber. In den Wasserleitungen des Hähnchenmastbetriebs konnten nach Reinigung und Desinfektion an allen Probenahmestellen *Pseudomonas aeruginosa* detektiert werden, von denen ein Großteil eine Antibiotikaresistenz aufwies. Ein Monitoring der Reinigungseffizienz und eine sorgfältige Schulung der Mitarbeiter können dabei helfen, den

Hygienestatus zu verbessern und die Ausbreitung pathogener und antibiotikaresistenter Bakterien zu verringern. Hierbei sollte besonderer Wert auf kritische Punkte wie Fütterungs- und Tränkeeinrichtungen gelegt werden.

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List of abbreviations

A. baumannii	*Acinetobacter baumannii*
ACP	Agar contact plate
ATP	Adenosine triphosphate
BMEL	Bundesministerium für Ernährung und Landwirtschaft
CD	Council Directive
cfu	colony forming units
CI	confidence interval
C. parvum	*Cryptosporidium parvum*
CR	Council Regulation
DE	Dey Engley agar
DLG	Deutsche Landwirtschafts-Gesellschaft
EC	European Community
E. coli	*Escherichia coli*
ELISA	enzyme-linked immunosorbent assay
ESBL	extended-spectrum beta-lactamases producing bacteria
ESKAPE	*Enterococcus* spp., *S. aureus, K. pneumoniae, A. baumannii, P. aeruginosa, Enterobacter* spp.
EU	European Union
EUCAST	European Committee on Antimicrobial Susceptibility
INCA	Infection Control Nurses Association
K. pneumoniae	*Klebsiella pneumoniae*
P. aeruginosa	*Pseudomonas aeruginosa*
MRSA	methicillin-resistant *Staphylococcus aureus*
OR	odds ration
PC	plate count agar

Reg.	Regulation
RLU	relative light units
RODAC	Replicate Organism Detection And Counting
S. aureus	*Staphylococcus aureus*
SchHaltHygV	Schweinehaltungshygieneverordnung
spp.	species pluralis
TCC	total coliform count
TierSchNutztV	Tierschutz-Nutztierhaltungsverordnung
TSE	transmissible spongiform encephalopathy
TVC	mesophilic aerobic total viable count
UV	ultraviolet
VRBD	violet red bile dextrose agar
VRE	vancomycin-resistant *Enterococci*

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1. Introduction

The term ‘hygiene’ is derived from the Greek ‘hygieinós’, which means ‘beneficial to health’ and includes a variety of measures. According to common dictionaries (Merriam-Webster, 2007; Duden, 2019), different definitions are given. For example, ‘Hygiene’ is used for a field of medicine or a field of science concerned with the maintenance and the promotion of health as well as its natural and social preconditions. Probably the most common meaning of hygiene is ‘conditions and practices of cleanliness conductive to health’ (Duden, 2019). Also in the field of animal husbandry, the term is used in textbooks with this topic for a variety of measures that might have an influence on farm animals’ health, such as the climate in stables, the quality of feed and water, or housing conditions. According to the International Society on Animal Hygiene, ‘animal hygiene’ is defined as follows: ‚the field of animal hygiene includes the interaction between abiotic and biotic factors of environment and domestic animals, especially food animals, with the aim to prevent diseases and to promote animal health and to ensure species-specific health and welfare needs of such animals’ (Thielen, 2000). In animal hygiene, therefore, the main objective is not to cure animals that are already diseased, but rather to provide preventive health protection and to create optimal environmental conditions in order to maintain a high standard of health and welfare (Hoy et al., 2016). The prevention of possible zoonosis also helps to protect the health of employees and to ensure a safe product at the end of the food chain, thus making an important contribution to work safety and consumer protection. Even though the aspect of animal hygiene covers a wide scope, the content of this thesis focuses primarily on the common use in terms of cleanliness, maintained by cleaning and disinfection, the implementation of hygiene management and suitable measures for success control of sanitation in livestock farming. The importance of cleaning and disinfection in livestock production increases with the rising numbers of animals per area and per barn (Müller et al., 2011). Therefore, sanitation is an integral part of modern intensive farm animal housing.

1.1. Legal requirements for sanitation

The legal requirements for the execution and frequency of cleaning and disinfection are regulated with varying degrees of severity depending on the considered farm animal species. In general, the farmer is given a relatively large scope of action, especially for managing hygiene measures in cattle and calves. In Figure 1.1 the legal framework on European and federal level dealing with hygiene in livestock farming, which includes mammals as well as poultry is shown.

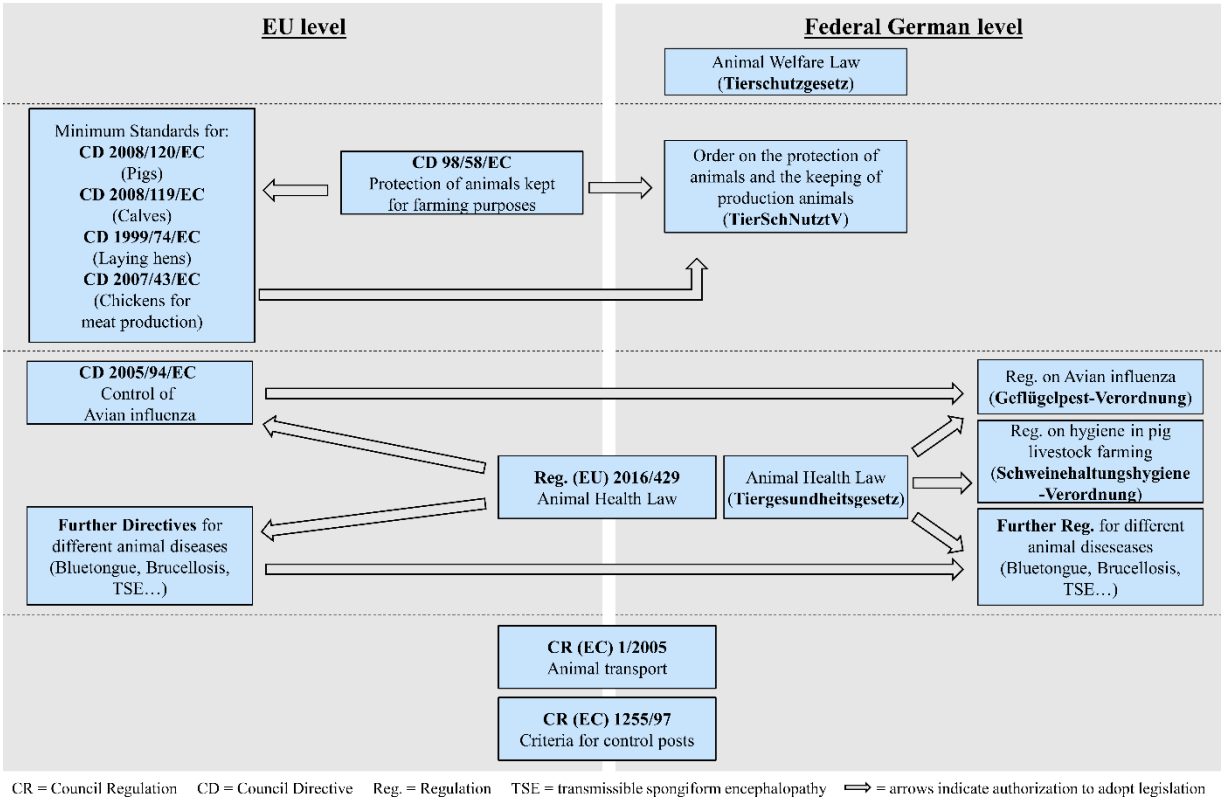

Figure 1.1. Legal requirements associated with hygiene in livestock farming at European and federal German level.

The legislation of the European Union differs in Regulations, which are binding legislative acts in all their parts and directly applicable by all member states. Furthermore, there are Directives, with defined objectives, which are to be achieved by all EU countries and which must first be devised into law by the member states (EU, 2019). In addition, there are laws that apply exclusively at federal German level, such as the Animal Welfare Law or the national Animal Health Law, which came into force in 2013 replacing the original national Animal Diseases Law of 1880. On the basis of the former German Animal Diseases Law further Regulations were issued, such as the Avian Influenza Regulation (originally from 1972), which additionally served to implement the Council Directive 2005/94/EC after revision. Because that the topic of animal health and hygiene has been an established part of the legislation for a long time and

since legislation is subject to constant change, especially due to harmonization acts by the EU, it is difficult to illustrate the currently valid legal framework and its origin.

1.1.1. Legal requirements at EU level

At EU level, the main rules with minimum requirements for animals bred or kept for farming purposes, including fish, reptiles and amphibians and excluding invertebrate animals, are laid down in the Council Directive 98/58/EC concerning the protection of animals kept for farming purposes (1998). It also authorizes the member states to take further measures to ensure that farmers can guarantee the welfare of their animals, which must not be subjected to unnecessary pain, distress or harm. In accordance with the Directive, the implementation of appropriate measures is transferred to the member states. These measures are monitored by veterinary experts of the European Commission. Specific requirements concerning cleaning and disinfection are laid down in the Annex to the Regulation, which explicitly does not apply to fish, reptiles and amphibians. Furthermore, materials to be used for the construction of livestock buildings and equipment with which the animals may come into contact must not be harmful and thoroughly cleansed and disinfected. Feed and water shall not contain substances that cause unnecessary suffering or injury and repositories for feed and water shall be designed to minimize possible contaminations. Animals must be provided with water of an ‘appropriate quality’. Also, dust levels and levels of emissions must be kept within limits that are not harmful to the animals. Besides this Council Directive, which is binding for the care of all animal breeds kept for farming purposes, the EU adopted additional Council Directives, laying down minimum standards for the protection of pigs, calves, laying hens and fattening chicken. The additional requirements relating to hygiene of these Directives, supplemental to those of CD 98/58/EC, are shown in Table 1.1. Currently, there is neither a comparable Council Directive concerning minimum standards for the protection of dairy cows, nor for beef cattle, which is strongly criticized by Nalon und Stevenson (2019). On EU level, a lack of minimum standards for farm animals of lesser economic importance, such as small ruminants, fattening rabbits and all kinds of poultry besides laying hens and fattening chicken, for example turkeys, ducks and geese, can be identified. According to these Council Directives, member states are authorized to determine stricter Regulations in consultation with the Commission.

Table 1.1. Requirements related to hygiene and evaluated gaps in requirements of the Council Directives (CD) 2008/119/EC, 2008/120/EC, 1999/74/EC and 2007/43/EC.

Reference	Requirements / Content	Evaluation / Gaps in requirements
CD 2008/120/EC laying down minimum standards for the protection of pigs	All pigs must have access to an adequately clean lying area. In case of earlier weaning of pigs (before 28 days of age), they must be moved in specialized housing facilities, which are emptied and thoroughly cleaned and disinfected before the introduction of a new group to minimize transmission of diseases. Pregnant sows and gilts must be thoroughly cleaned before they are placed in farrowing crates.	Requirements about routine cleaning and disinfection, with regard to early weaned pigs, are missing. The term 'thoroughly' seems rather subjective. A description of how cleaning of pregnant sows and gilts should be performed is not given.
CD 2008/119/EC laying down minimum standards for the protection of calves	This CD clearly states that housing pens, equipment and utensils used for calves must be properly cleaned and disinfected and that feces, urine and uneaten or spilt food must be removed as often as necessary.	Adequate time intervals to carry out these measures are missing.
CD 1999/74/EC laying down minimum standards for the protection of laying hens	After every depopulation and before new hens are brought in, the stable and parts of equipment with direct contact to hens must be thoroughly cleansed and disinfected. During occupation of cages, all surfaces and equipment must be satisfactorily clean. Additionally, feces must be removed as often as necessary and dead chickens must be removed daily.	A more precise description of the term 'satisfactorily clean' is not provided. An adequate time interval for removing feces is missing. The term 'thoroughly' seems rather subjective. Cleaning and disinfection measures are limited to areas and equipment with direct animal contact.
CD 2007/43/EC laying down minimum standards for the protection of chickens kept for meat production	After every depopulation and before new hens are brought in, the stable and parts of equipment with direct contact to animals must be thoroughly cleansed and disinfected. When all chickens are removed from the pen, the used litter must be removed, and the barn must be equipped with clean litter.	Cleaning and disinfection measures are limited to areas and equipment with direct animal contact.

Further legal requirements at EU level widely dealing with hygiene, govern the handling of animal diseases (Reg. (EU) 2016/429, "Animal Health law"), the protection of animals during transport (Reg. (EC) 1/2005) and criteria for control posts (Reg. (EC) 1255/97). On the basis of the Regulation (EU) 2016/429, other Directives have been adopted which deal specifically with individual animal diseases, like avian influenza (Reg. (EC) 2005/94).

1.1.2. Legal requirements at federal level

In Germany, the highest legal principle for the handling of animals is the Animal Welfare Law (Tierschutzgesetz, 1972). According to § 2 No 1, everyone who keeps, cares for or has to care for an animal must feed, maintain and house the animal in a manner appropriate to its species and needs. This includes a clean environment, which should not enhance the development of diseases. Specific hygiene requirements for livestock farming are laid down in the German Order on the protection of animals and the keeping of production animals (Tierschutz-Nutztierhaltungsverordnung, TierSchNutztV, 2001), which came into force on 01 November 2001 and was last amended on 30 July 2017. It transposes the relevant European Directive 98/58/EC, as well as the European Directives 2008/119/EC, 2008/120/EC, 1999/74/EC, and 2007/43/EC for the individual livestock animal species into national German law. The hygiene requirements are therefore largely the same as those specified in the respective EC Regulations, therefore only additional Regulations are mentioned below. The scope of the German Order on the protection of animals and the keeping of production animals is restricted to animals for commercial purposes, which excludes, for example hobby animal husbandry. This Order first lists criteria that apply to all farm animals and presents further specific Regulations for the farm animal species: calves, laying hens, fattening chicken, pigs, and additionally for rabbits. With regard to hygiene according to § 4 (1) No 10, in accordance with Annex I of the CD 98/58/EC, it applies that people who keep farm animals must ensure that the housing facilities are kept clean, in particular that excrements are removed as often as necessary and that parts of buildings, equipment and utensils with which the animals come into contact are cleansed and disinfected at appropriate intervals. The interpretation of what is considered to be an ‘appropriate interval’ and why it excludes cleaning and disinfecting of areas such as surfaces of feeding or drinking tubes and ceilings without direct animal contact, although transmission pathways and interactions are scientifically proven (Stärk, 1999; Zhao, 2011), is uncertain. An additional requirement is noted for the keeping of pigs: the farmer has to make sure that farrowing pens are cleaned prior to housing of pregnant sows. For the keeping of rabbits, the farmer must ensure that part of any housing facilities, equipment or utensils in contact with the rabbits are cleaned and disinfected after every depopulation, which also applies, in accordance with EU and federal legislation, to laying hens and chickens for meat production. A violation of the prescribed cleaning and disinfection procedures after the depopulation is considered an administrative offence for laying hens and fattening chickens, but not for rabbits.

The national German Animal Health Law (Tiergesundheitsgesetz, 2013), which was derived from the original national German Animal Diseases Law, is designed to prevent and control animal diseases. It also intends to maintain and promote the health of farm animals. This law is primarily the basis for authorization to create further Regulations for specific animal diseases and defines the authorities' scope of action. Based on the national German Animal Health Law, the ordinances against bluetongue disease, brucellosis, transmissible spongiform encephalopathy (TSE) and avian influenza, as well as the Livestock Traffic Regulation and the Regulation on Hygiene in pig livestock farming were issued. Most of these Regulations apply exclusively in case of an infectious disease outbreak and contain specific rules for dealing with infected animals in order to prevent the spread of specific infections.

The national German Regulation on hygiene in pig livestock farming (Schweinehaltungs-hygieneverordnung, 1999) lays down different hygiene requirements for the farmer depending on the size of the pig holding. At all farms, regardless of their size, the herd must be supervised by a veterinarian. This includes advising the farmer with the aim of maintaining or, if necessary, improving the herds' health status. In line with the inherent concept of quality management, a continuous improvement of the health status would be preferable in order to maintain the standard. To ensure a high standard, the veterinarian must provide evidence of further training at regular intervals, which have to cover, in particular, farm hygiene measurements. In addition, the Regulation on hygiene in pig livestock farming lists requirements with regard to vector control, such as rodent control, hygiene of employees, facilities and objects. Hygiene measures for people include the presence of hand washing basins, the wearing of protective clothing as well as the cleaning and disinfection of footwear. Hygiene measures for facilities and objects cover the cleaning and disinfection of stables, pens, buildings, transport vehicles, and loading facilities, in general. Interestingly, the requirement for cleaning and disinfection after depopulation of the compartments in which the pigs are kept applies only to farms with more than 20 rearing or fattening places and breeding or mixed holdings with more than three places for breeding sows, but not to smaller holdings. The liquids produced during cleaning and disinfection must be disposed of without causing damage. According to common practice in pig production, these liquids flow into the manure pit due to widespread installment of slatted floors and are further processed together with the slurry.

The national German Regulation on Avian influenza (Geflügelpest-Verordnung, 2007) is based on the Reg. 2005/94/EC, which contains very precise instructions on hygiene in the case of disease outbreak. The general requirements, which apply irrespective of a disease outbreak, are

set out below. It aims primarily at farm sizes of more than 1,000 animals. Furthermore, regardless of the occurrence of diseases, it also contains general rules on the use of protective clothing, cleaning and disinfection of farm vehicles, equipment, loading areas and stables, including the facilities and objects present in avian production. This, therefore, does not only apply to objects with which animals were directly contacted. Each poultry farm must also have facilities for washing hands, changing clothes and disinfecting footwear. Containers for collecting carcasses must be cleaned and disinfected after each disposal by a knacker, but at least once a month. Even stricter prescriptions apply in the case of suspected avian influenza.

1.1.3. Recommendations and guidelines

Various practical guides or information sheets have been published by different associations that deal with hygiene management in livestock farming. For example, the DLG (Deutsche Landwirtschafts-Gesellschaft) has published a guideline on hygiene technology and management instructions for cleaning and disinfection of livestock buildings, which describes in detail the procedure of sanitation, including frequent mistakes and how these can be prevented (Von der Lage et al., 2010). Another DLG publication deals specifically with biosafety in cattle farming and points out, among other things, weaknesses in hygiene (Münster et al., 2018). For cattle and small ruminants, the Federal Ministry of Food and Agriculture (Bundesministerium für Ernährung und Landwirtschaft, BMEL) has also published a recommendation for hygienic requirements for the keeping of ruminants (BMEL, 2014). Further sources of information for farmers are often consultants for cleaning and disinfection products, trade fairs like ‘Agritechnica’ or ‘EuroTier’, internet references, and specialist journals that deal with the topic hygiene management at regular intervals and sometimes publish special issues on the subject, such as the DGS, specifically for poultry or the TopAgrar.

1.2. The role of hygiene in farm animal housing

It is generally known that hygiene in farm animal housing is important for the performance and health of the animals (Hoy et al., 2016). However, that the topic remains highly relevant can be seen from the frequency of various hygiene topics in the agricultural literature. By reviewing specific agricultural journals for farmers (from January 2017 until April 2020), keywords related to hygiene in livestock housing appeared 110 times in the headings (Fig. 1.2). The total number of keywords found depends on the frequency of publication, volume and style of the magazines. For example, headings like ‘Unsexy, but indispensable’ (‘Unsexy, aber

unverzichtbar', ELITE, 01/2018) or 'Two just in case' ('Zwei für alle Fälle', TopAgrar, 07/2019) do not indicate whether the content of the article deals with hygiene or a completely different topic and were neglected. Therefore, the absolute number of articles dealing with hygiene might be higher.

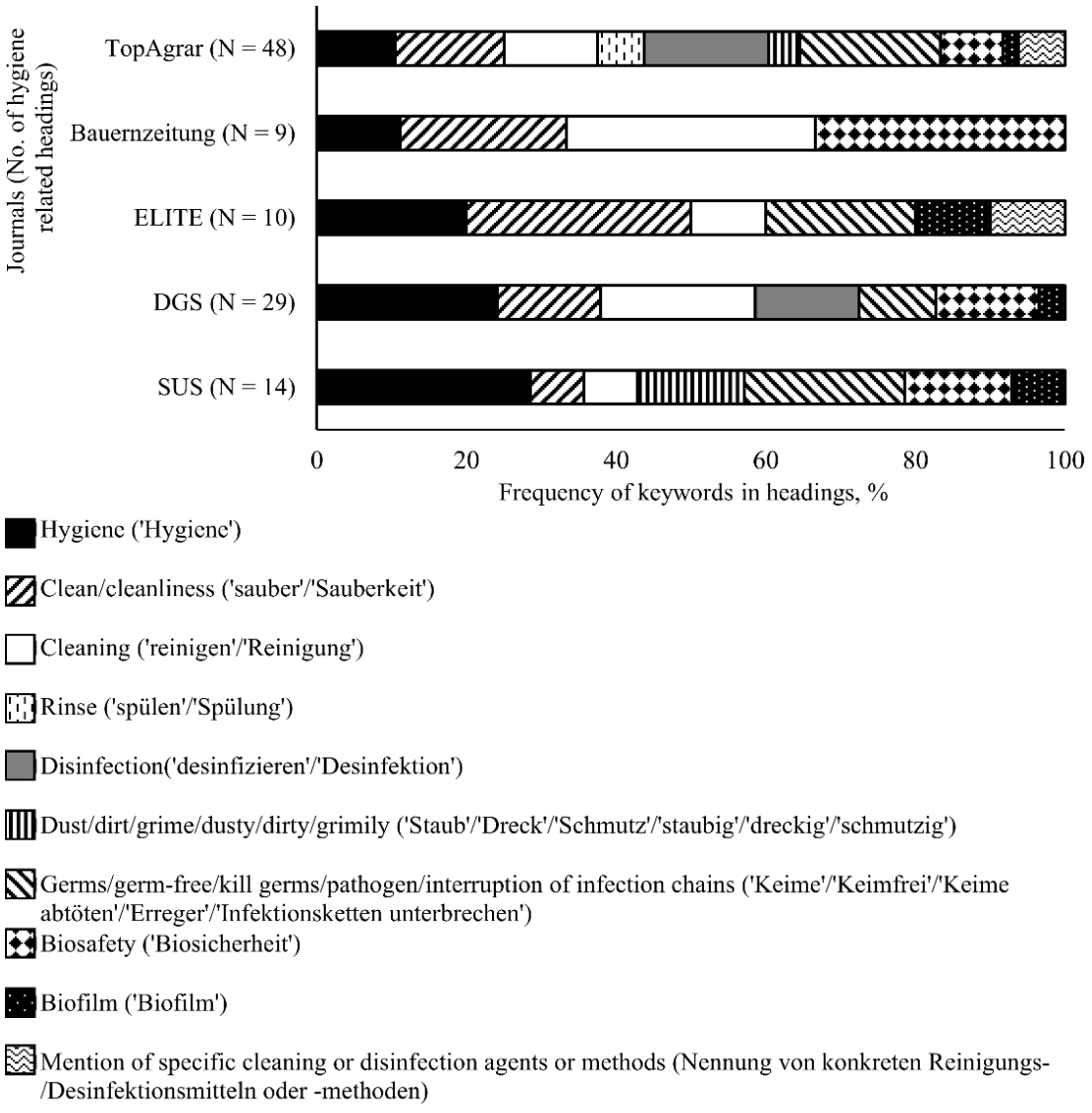

Figure 1.2. Frequency of hygiene related headings generated by a keyword search in two journals with general agricultural topics (TopAgrar and Bauernzeitung) and three journals with specific animal species topics (ELITE for cattle, DGS mainly for poultry, and SUS for pigs), published from January 2017 until April 2020. Keywords are ranked from general to specific terms.

When searching for recommendations on the proper implementation of hygiene measures in agriculture via the internet search engine 'Google', it becomes obvious that the advertising and contributions from cleaning and disinfectant manufacturers dominate the generated results. This is especially true for a search in German combining the term 'stable' with the keywords 'hygiene' (58% of all hits are advertisements), 'cleaning' (69% advertisements) or

'disinfection' (73% advertisements). By adapting the keywords (e.g. addressing certain animal species instead of the term stable), a better hit rate for non-commercial information sources such as public institutions or associations can be achieved. As an example, Figure 1.3 shows the first 45 hits for a search via the internet search engine 'Google' with several hygiene related keywords. Of course, it must be taken into account that search algorism is adapted to the user and therefore deviations may occur depending on the user's search history. For the shown search run, an account was used from a person, who does not primarily work in science, but with an agricultural background in order to mimic a configuration and user behavior as expected for farmers.

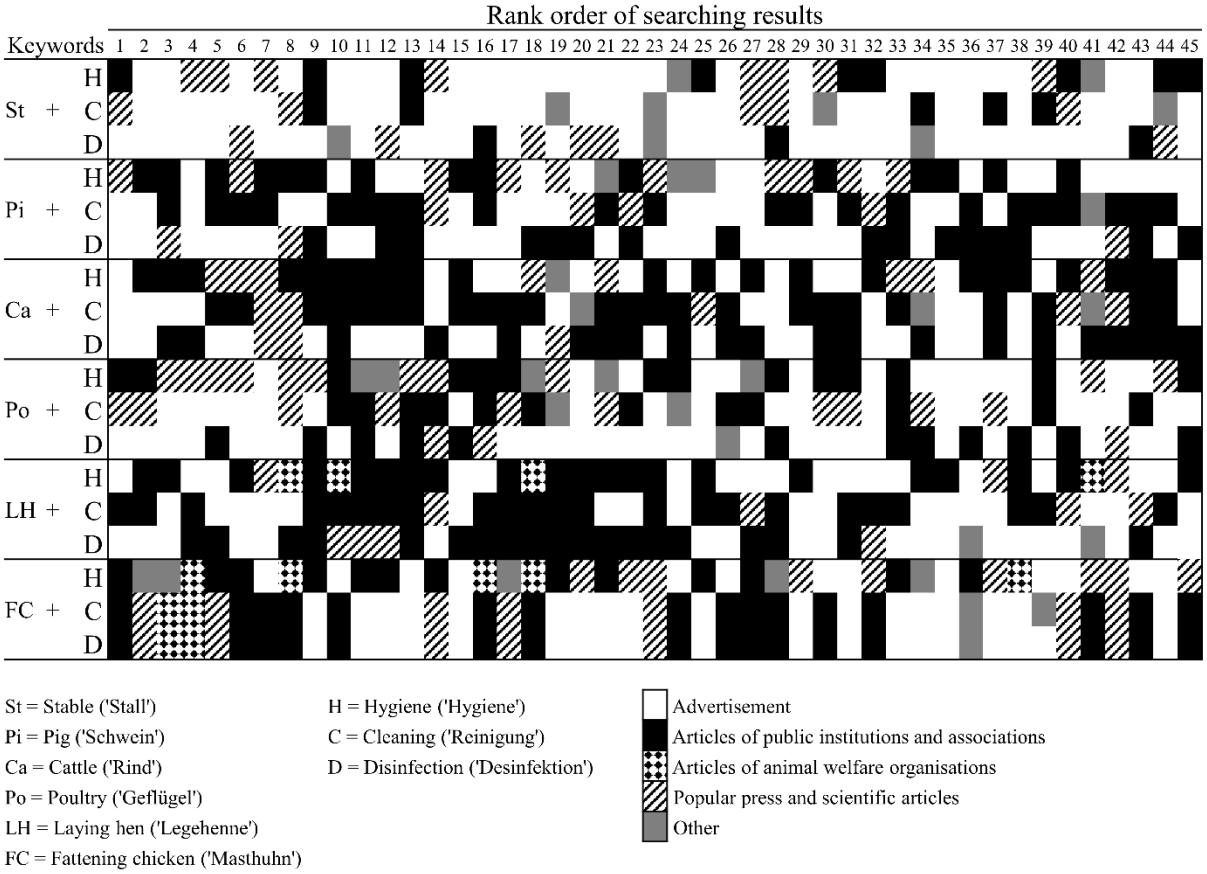

Figure 1.3. Evaluation of the information sources of the first 45 Google hits for three selected hygiene related keywords combined with the general term 'stable' or different farm animal species.

For neutral information on the correct procedure for cleaning and disinfection in stables, which is not driven by commercial interests, it is therefore advisable to refer to the mentioned guidelines from official bodies. The following procedure is commonly recommended for cleaning and disinfection in livestock buildings: first, coarse dirt such as manure and litter should be thoroughly removed from the building by scraping and sweeping. This is followed by a soaking phase, with the addition of cleaning agents, especially in the case of dirt with high

fat content, and subsequent cleaning with a high-pressure cleaner. After a sufficient drying phase, a prophylactically disinfection is carried out. It is further recommended to leave the pens or stables empty for 4 to 5 days (Von der Lage et al., 2010). A vacant time between animal batches reduces the microbial burden in the environment, such as the amount of inhalable dust and concentrations of airborne bacteria (Banhazi & Cargill, 1998). Routine disinfection in livestock farming is called 'prophylactic disinfection'. Its aim is to keep the load of pathogenic bacteria below the infectious dose. Prophylactic disinfection is most effective when carried out in a completely empty stable (Müller et al., 2011). The possibility of implementing this procedure depends strongly on the respective livestock species and the particular production stage. At production stages that strictly follow the all-in-all-out principle, the individual process steps can be integrated into hygiene management without difficulty. At production stages with continuous occupancy the implementation is more challenging, since most disinfecting agents can have negative impacts on animal health through inhalation or skin contact and should therefore not be applied during animal stocking. Additionally, the recommended times for drying and exposure to disinfectants may not be maintained.

In conventional pig husbandry, the all-in-all-out principle is well established on most production stages (Fig. 1.4). Only the keeping of boars and pregnant sows in the gestation unit is more often done in a continuous process, depending on the size of the farm. It is also common practice to move the animals to new pens during fattening, for example to reduce the group size and adapt to the increasing space requirements of growing pigs. Pigs on the flat deck and in fattening pens, whose weight deviates too much from the group mean due to a reduced weight gain, are usually integrated into other groups. This switching of groups however might represent a threat to the all-in-all-out principle. The individual production stages can be combined in a closed system on one farm. However, farms often have only one or more specialized production stages such as farrowing, piglet rearing and fattening. Accurate housing hygiene is particularly important when housing pigs purchased from another farm, as these must adapt to the farm-specific bacterial community first (Fotheringham, 1995). Therefore, purchased gilts are usually first kept in isolation units before they are accustomed to the bacterial flora of the farm in inclusion pens through contact with old sows or weaned piglets.

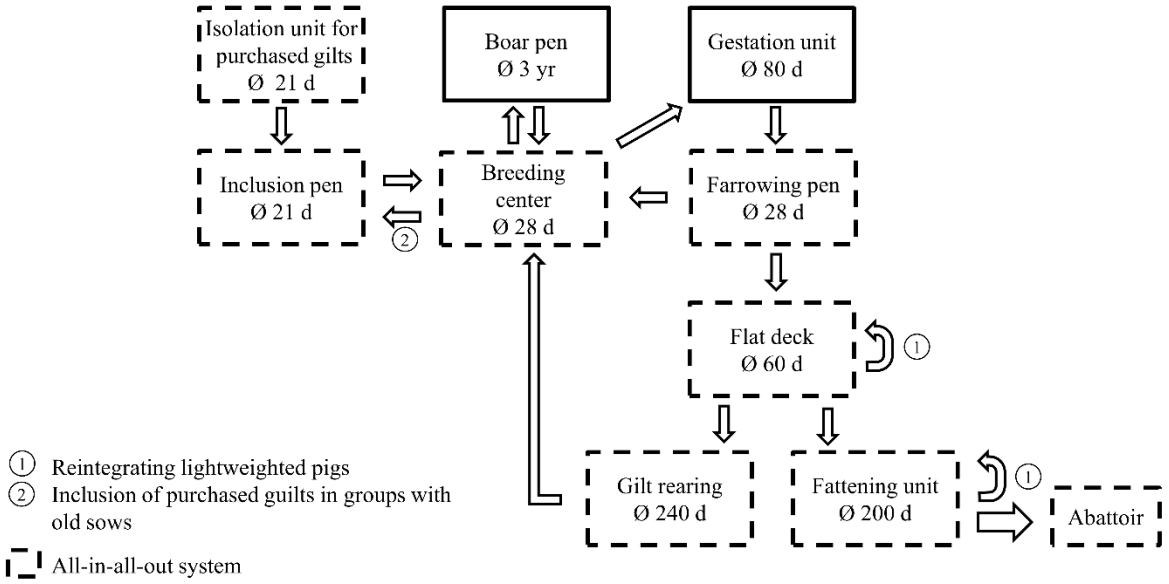

Figure 1.4. Different stages of a common pig production cycle with average occupancy time. Composed of data from Sommer et al. (1991), Weiß et al., (2000), Tsakmakidis et al. (2012), and Hoy et al. (2016).

In dairy farming, it is not easy to establish a uniform scheme comparable to pig production because of the many different types of farming (Fig. 1.5). Both the farming methods and the average occupancy times differ greatly depending on the individual farms and show a high variation. In cattle fattening there are differences depending on whether calves, oxen or heifers are fattened for meat production. Besides non-rentable older dairy cows and fattening cattle, cows from all production stages can be sent to the abattoir due to health or fertility problems. The production cycles for keeping dry cows, lactating cows in the dairy stable and heifer rearing usually undergo continuous processes. All-in-all-out methods are mostly implemented in maternity pens, calf housing (single and group) and cattle fattening. It should be noted that for cattle fattening animals are purchased from different farms meaning that all are colonized with a varying composition of bacterial species and potential pathogens. Particularly at localities of calf or cattle livestock traders, a large number of animals from different farms come together, which enhances an exchange of pathogens.

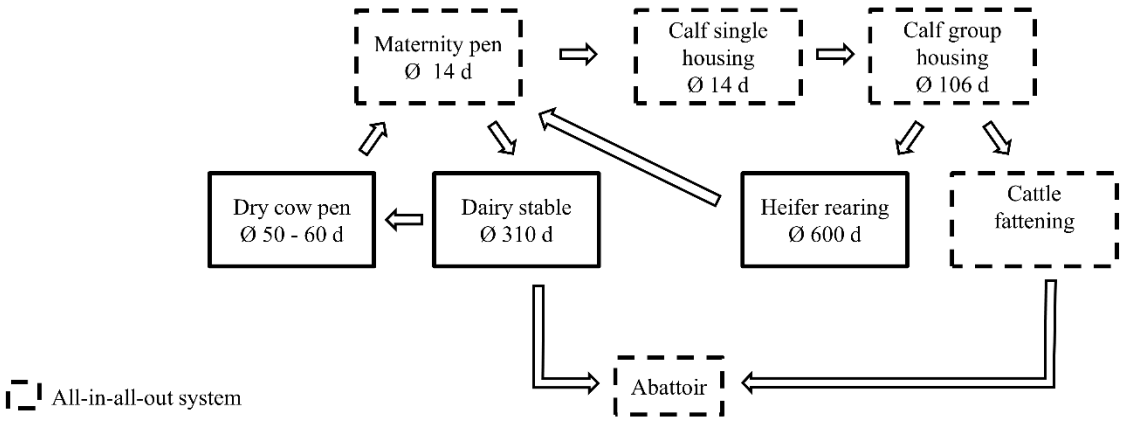

Figure 1.5. Different stages of a common dairy production cycle with average occupancy time. Composed of data from Sommer et al. (1991), Weiß et al., (2000), and Hoy et al. (2016).

In the conventional poultry production, all stages of production are strictly based on the all-in-all-out method, which facilitates compliance with the hygiene measures prescribed by law (Fig. 1.6). To minimize the transmission of diseases, the principle of dislocation is applied in poultry farming. For this purpose, the distances between large flocks should be 1,500 (in forest areas) or 3,000 m (in open areas). Between individual production units, there should be a distance of 200 to 500 m and between the stables of one production unit a distance of 10 to 20 m (Müller et al., 2011). The individual production stages are usually carried out on separate specialized farms. For restocking, there is no mixing of hatchlings from different origins. However, there are great differences in the length of the different production stages. In chicken fattening, an average production cycle is completed after a period of 28 to 42 days, depending on the type of fattening. The production cycles in breeding companies or in layer houses are considerably longer at an average of 420 days or 500 to 600 days, respectively, which leads to the creation of a hygienically precarious milieu (Müller et al., 2011). As a result, the risk of infections can increase due to an expanded bacterial flora.

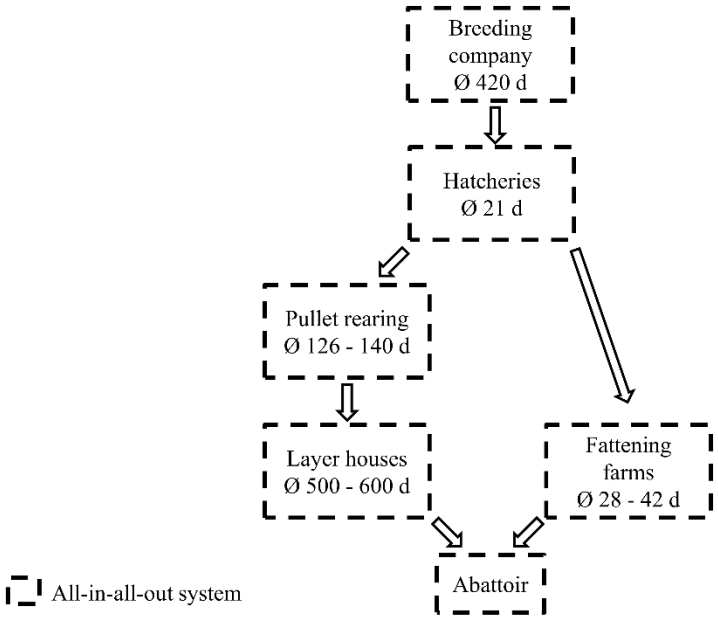

Figure 1.6. Different stages of a common chicken production cycle for laying hens and chicken for meat production with average occupancy time. Composed of data from Weiß et al. (2000), Damme & Hildebrand (2002), and Hoy et al. (2016).

In general, however, when comparing the different farm animal species, it can be stated that especially keeping of young animals is based on the all-in-all-out production principle. As young animals are more sensitive to disease due to their insufficiently developed immune system, special attention should be paid to the hygiene of their pens, as well as to the housing- and feeding equipment of the young animals in order to avoid a vertical spread of infectious agents (Fotheringham, 1995).

1.3. Hygiene indicators in the hospital sector and food industry

To evaluate the success of hygiene measures for housing- and feeding equipment in farm animal husbandry, appropriate methods are necessary. There are various procedures for assessing cleaning and disinfection, which are already routinely used in the hospital sector and food industry and occasionally in farm animal housing.

1.3.1. Visible evaluation

The easiest and most cost-effective, but also most inaccurate method to check the success of cleaning and disinfection is a visual evaluation. In addition, cleanliness can also be checked haptically by touching the surface, as clean surfaces should feel smooth and free of irregularities. In hospital settings, sometimes chemical tracers are used, which fluoresce under UV light and can be easily removed by wet mopping. Different sampling spots are marked with this substance prior to cleaning. An ultraviolet (UV) lamp can be used to check if the marks were removed during cleaning. For evaluation, results are distinguished in clean (complete or partial remove of the mark) and dirty (mark is not removed; Carling et al., 2006; Goodman et al., 2008). Further advantages of this simple method are that results are immediately available and follow-up measures such as a renewed cleaning, can be undertaken immediately (Schmitt & Moerman, 2016). The disadvantage lies in the subjective assessment of cleanliness (Osimani et al., 2014): If the same person carries out both sanitation and following visual inspection, it is likely that areas that have been skipped at cleaning will probably be forgotten during inspection as well. Moreover, the visual cleanliness of a surface displays only limited information about its microbial status. Even on surfaces that appear clean, pathogenic microbes might still be present (Lewis et al., 2008). In the food industry, surfaces that appear visibly clean can still contain 10^7 to 10^8 cfu (colony forming units) $\cdot$ cm^{-2} (Weber, 1996). For assessing the cleanliness of surfaces, scales with a varying number of degrees and different evaluation criteria are used. In several studies evaluating the cleanliness in poultry houses, a three-level scale (from 0 to 2) is chosen, with the evaluation criteria: 0 = dirty; 1 = not completely cleaned, traces of dust, feathers, egg or manure; 2 = clean (Huneau-Salaün et al., 2010; Luyckx et al., 2015). Without a more precise definition of the term ‘dirty’, the assessment remains rather subjective. In studies from the hospital sector, the scaling for the visible evaluation of cleanliness varies greatly. In a study by Mulvey et al. (2011), cleanliness is rated on a scale of 1 to 10, with 1 = unacceptable und 10 = clean. For evaluation, a checklist was used, noticing visual dirt, rubbish, smears, dust, grease, fingerprints and other. Sherlock et al. (2009) probably used a two-step scale for assessment of visual cleanliness. Precise details are lacking, but it is mentioned that the term ‘clean’ is based on the absence of visual soiling, presence of moisture, staining or poor surface condition. The study from Sherlock et al. (2009) also refers to the audit guidelines of the Infection Control Nurses Association (INCA). A number of other studies also fail to provide a precise procedure for assessment. Griffith et al. (2007) and Lewis et al. (2008) mention that the assessment of cleanliness is based on the degree of staining, the presence of visual organic soil,

and the presence of moisture. In both publications, the same primary source is cited for the standardized methodological procedure, in which only the following sentence is noted: ‘Surfaces were visually assessed for general condition, cleanliness and moisture using a standardized proforma’ (Griffith et al., 2000). Frequently, in studies from the hospital sector the visual result is expressed as a grading sum of individual points in the sampled ward. It is therefore not surprising that in these studies from hospital settings often no correlations are found between total visual evaluation and the cleanliness of specific single objects generated by microbiological or other methods. Nevertheless, visual assessment is generally recommended and commonly used as the primary method for the evaluation of sanitation’s effectiveness in food industry, healthcare and agriculture (Huneau-Salaün et al. 2010; Cunningham et al., 2011; Osimani et al., 2014; Mitchell et al., 2015).

1.3.2. Protein rapid tests

Conventional rapid tests for detection of remaining protein residues are characterized by chemical reactions, where remaining proteins are indicated by a color change. There are several suppliers for such test systems, like Hygiena (PRO-Clean), Merck (FLASH Rapid Allergen – Protein Detection Test), SP Medikal (Protein Rapid Swab Test) or 3M (Clean-Trace Surface Protein Plus). For sampling with these tests, a defined area is swabbed to collect possible protein residues from surfaces. Then the swab is well shaken with reaction solutions to dissolve the protein residues and initiate the chemical reaction. The resulting color change allows semi-quantitative interpretation of the content of protein residues after a defined time interval (for example ten minutes, depending on the manufacturers’ guideline). Such rapid tests are frequently used to monitor the cleanliness of food equipment or medical devices. Most of these tests are based on the so-called biuret reaction, in which peptide bonds of proteins form a mauve colored complex with copper (II) ions in the alkaline. There are different modifications of the assay, like the bicinchoninic acid assay and the Lowry assay. In both assays, further reactions of the copper ions with different reactants are intended to intensify the color change and increase the test´s validity (Smith et al., 1985). The evaluation performed with these test systems is usually semi-quantitative by comparison with a color scale (Fig. 1.7).

Color	Detection range	Description of test result	Interpretation of test result
1	~ 0 - 10 µg	No color change	Highly satisfactory clean
2	~ 10 - 30 µg	Light color change	Satisfactory clean
3	~ 30 - 60 µg	Color change to gray	Visible control necessary
4	~ 60 - 300 µg	Color change to light purple	Unsatisfactory clean
5	> 300 µg	Strong color change to purple	Highly unsatisfactory clean

Figure 1.7. Color change scheme, detection range and criteria for assessment of the protein rapid tests used based on own studies.

The test results can be affected by other reducing components such as uric acid and reducing sugars, like glucose. For assessment of cleanliness in the livestock sector, cross-reactions with these components are no obvious disadvantages, as all other substances should be removed after sufficient cleaning. According to manufacturer specifications, the lower detection limit is 20 µg protein at room temperature or 10 µg protein at 37°C. To clarify the possible usage of protein tests it should be noted that this test can make statements about the cleanliness of a surface and possible residues, but gives no information on remaining microorganisms or survival.

1.3.3. Adenosine triphosphate (ATP) rapid tests

The basis of commercially available ATP rapid tests is an enzymatically bioluminescence reaction in which the enzyme luciferase originating from fireflies as a catalyst leads to an oxidative decarboxylation of luciferin promoted by ATP, as is shown in the equation below (Hawronskyj & Holah, 1997). ATP is the main energy carrier in all cells of plants, animals and microbes. Remaining soiling in farm animal housing usually consists of feces, urine, feed residues, animal cells and bacteria, which is why the content of measured ATP can be used as an indicator for hygiene. During the reaction light is emitted, which can be measured with a so-called Luminometer. The amount of emitted light is expressed in relative light units (RLU) and is proportional to the amount of ATP residues (Amodio & Dino, 2014)

$$\text{D-Luciferin} + O_2 + \text{ATP} \xrightarrow{\text{Luciferase}} \text{Oxyluciferin} + CO_2 + \text{AMP+PP} + \text{light}$$

The collection of ATP residues is also performed with a swab by swabbing a previously defined area. The reaction is started by shaking the swab together with the reaction solutions. After an incubation time of 15 seconds, the result is available, expressed as a number in RLU. For regular

use in hygiene assessment, a defined ATP cutoff value and perhaps the preparation of a quality control chart is recommended (Turner et al., 2010; Osimani et al., 2014). Since the requirements for cleanliness and what is considered a tolerable residual ATP cutoff value depend strongly on the industrial sector (hospital vs. food industry vs. agriculture) and within this sector on the respective sampling point (e.g. feeding equipment vs. floor) different cut-off values should be defined prior to sampling. There are different manufacturers for commercially available ATP rapid tests like HyServe (PD-10), Merck (HY-LiTE), 3M (Clean-Trace), Hygiena (Ultrasnap), Charm Sciences (PocketSwab Plus) or Kikkoman (Luci-Pac). Various studies conclude that results of the test systems from different brands are not comparable and show differences in detection limits, which can lead to confusion, as all test results are given in RLU (Andersen et al., 2009; Omidbakhsh et al., 2014; Whiteley et al., 2015). Therefore, if a system for ATP measurement has once been implemented and specific limits for each sampling point have been defined, there should be no switching between test systems from different manufacturers. Residues of detergents or disinfectants can affect ATP measurements by quenching or enhancing the emitted light intensity (Velazquez & Feirtag, 1997). Most of routinely used chemicals lead to a decrease in ATP values, like residues of foaming acid, alkaline cleaner-degreaser, chlorinated alkaline cleaner, acid sanitizer, iodine cleaner-disinfectant, acidic peroxigen sanitizer and chlorinated sanitizer. An increase in ATP results was reported for residues of quaternary ammonium compounds used in disinfectants and laundry chemistries and tri(butoxyethyl)phosphate, a commonly used plasticizer (Green et al., 1999; Brown et al. 2010; Omidbakhsh et al., 2014). It should be mentioned, that ATP systems cannot detect spores, viruses or prions, as they do not contain ATP (Hansen et al., 2008, Alfa et al., 2015). Additionally, users should be aware, that the ATP value does not necessarily correlate with the microbial load of surfaces, even if only bacteria are measured. This is due to the fact that bacterial species vary in the amount of production and release of ATP in a given time and that naturally occurring soil is composed of different sources of ATP (Cunningham et al., 2011; Omidbakhsh et al., 2014; Öz & Arun, 2019)

1.3.4. Microbiological tests

Pertinent target organisms

In most microbiological examinations of the hygienic status of surfaces, the mesophilic aerobic total viable count (TVC) is measured. This indicates the total number of aerobic and facultative anaerobic germs on the examined surface. It does not distinguish between certain bacterial species, yeasts and molds. In the food industry, *Enterobacteriaceae*, *Escherichia coli*,

Salmonella spp. and coagulase-positive *Staphylococci* are also frequently examined beside the TVC. For these parameters, limit values for the determination of process hygiene are also listed in the Commission Regulation (EC) No. 2073/2005 on microbiological criteria for foodstuffs. In the hospital sector, in addition to the TVC the focus is on 'typical' healthcare associated bacteria such as *Enterobacter* spp., *Staphylococcus aureus* methicillin-resistant *S. aureus* (MRSA), or Enterobacteriaceae (Lewis et al., 2008, Sherlock et al., 2009, Casini et al, 2018). In studies to monitor the success of hygiene measures in livestock farming, besides the TVC the content of *Enterobacteriaceae* and total coliform count (TCC) is often measured, as both are indicators of fecal contamination. Furthermore, testing for *Salmonella* spp., *E. coli*, *Campylobacter* spp., *Enterococcus* spp., and *S. aureus* is frequently performed (Mannion et al., 2007; Klein et al., 2013; Luyckx et al., 2015).

Sampling of surfaces: Agar contact plates vs. swab samples vs. sock samples

For determining the microbiological contamination of surfaces, two different methods are commonly used: the so-called contact method with agar contact plates (ACP) and the swab method. Another, less frequently used, method in the food sector is the flooding method, in which the object to be examined is immersed and moved in nutrient solution to wash off adhering microorganisms. This method is not practicable for the investigation of hygiene in livestock sector with only a few exceptions: It might be applicable to small equipment items such as artificial teats, toys or manipulable material. For the contact method, a solid culture medium is pressed with light pressure onto the surface to be examined. The germs remain largely attached to the surface of the culture medium, which is then incubated and the developing colonies are counted afterwards. Frequently used systems for the contact method are so-called RODAC-Plates (Replicate Organism Detection And Counting) or dipslides. In RODAC plates, the agar is poured into a petri dish in such a way that the hardened culture medium protrudes convexly over the edge of the petri dish. Dipslides usually have a protruding culture medium applied to a plastic carrier from two sides. The choice of the culture medium depends on the expected bacterial species. The advantage of dipslides compared to RODAC plates is that they can also be used for immersion analyses by simply dipping them in a liquid sample and, after a short drip-off, screwing them back into the corresponding transport cylinder (Pichhardt, 1998). The advantages of agar contact plates are the simplicity in use, their portability and the absence of laboratory manipulation after sampling (Huneau-Salaün et al., 2010). One disadvantage of the contact plate method is, however, that other particles from the sampled surface besides the bacteria adhere to the culture medium, too. In addition, also from

easily accessible surfaces only a proportion of the microorganisms adhere to the contact plated. Moreover, curved or angled surfaces cannot be sampled due to their rigid shape. Application on rough surfaces can lead to destruction of the contact plate. The method is therefore only suitable for smooth, dry surfaces without excessive curvature or scratches.

An alternative technique, which is frequently used, is the swab method. Using this technique, the sample area is marked with a sterile frame. The marked area is wiped horizontally and vertically under rotation with a swab, which may have been moistened with sterile saline solution previously. The swab is then transferred to a suitable transport solution, such as Amies medium, and further processed in the laboratory. By thorough shaking with a vortex the bacteria are transferred into the liquid, from which, if necessary after further dilution, bacterial species are examined by means of plate pouring or spread plate techniques. Depending on the issue of the analysis, selective or non-selective culture media are used for cultivation (Pichhardt, 1998). The advantages of this method are that it is independent of moisture, adhering dirt and dust and the entire bacterial content. In addition, swabs can also be used to sample areas that are difficult to access, such as the inside of artificial teats or pipes. Reproducibility is, however, often criticized in swabbing procedures, as it is highly dependent on the user.

In agriculture, the so-called boot sock or boot swab sample method is the recommended method for examining the occurrence of *Salmonella* spp. in poultry houses (CR (EU) No 200/2010). According to this method, sterile disposable hairnets or similar cotton covers are pulled over disinfected clean shoes and a defined number of steps is taken through the barn. The hairnets are then transferred into sterile boxes with a liquid transport solution and can be further analyzed in the laboratory. In addition to the initial detection of *Salmonella* spp., this method is also used in other studies to determine *Campylobacter* spp. or MRSA (Berghaus et al., 2013; Friese et al., 2013).

Further sampling possibilities: manure, feed, litter, water, dust and air

In addition to sampling of surfaces, samples of liquid manure or feces, feed, litter, animal drinking water, stable air and dust can be collected for microbial examination for assessing hygiene in animal's housing and surrounding. However, manure and feces analyses are primarily used to check for the presence of certain pathogens such as bacteria or parasites in suspected cases and are therefore not the focus of this thesis (Sommer et al., 1991).

Conclusions about the quality of feed and litter can be drawn from an examination prior to feeding and are important, since improper storage can lead to a recontamination with spoilage

agents, pathogenic germs such as *Salmonella* spp. or molds, even if the products have been produced under optimal conditions (Sommer et al., 1991). According to Müller et al. (2011), most infectious agents can be transmitted to animals via feedstuffs.

Microbial examination of animal drinking water and stable air has long been known as an effective precautionary measure for disease prevention (Sommer et al., 1991). The quality of the drinking water primarily depends on the composition of the incoming water. Nevertheless, bacteria can migrate into the pipes due to close animal contact, therefore a differentiation is usually made between the water quality in the supply system and the quality of ingested water at the drinker (Van Eenige et al., 2013). Bacteria in water pipes usually form biofilms within a very short time. These biofilms are a conglomeration of different microorganisms in extracellular polymers developed by the bacteria and constitute a very complex community of single species or different genera (Costerton, 1995, Aguilar-Romero et al., 2010). In addition to relatively harmless environmental bacteria, pathogens can also accumulate in the biofilm and might continuously lead to health problems for the housed animals, if the biofilm is not removed thoroughly during cleaning and disinfection of water pipes (Wingender & Flemming, 2011). The formation of biofilms in milking systems and the difficulty of removing them solely by chemical agents in cleaning-in-place techniques has already been addressed in several studies (Mosteller & Bishop, 1993; Cherif-Antar et al., 2016; Weber, 2019). The mechanism of biofilm formation can also be transferred to similar systems, such as automated calf feeders and pipes of liquid feeding systems. The high nutritional supply in milk, milk replacer or feed intensifies this process. Water samples are taken directly from the trough or pipes, and depending on the issue, the stagnation water is first drained off. The microbiological examination is carried out directly from the water, or after dilution, if necessary (e.g. for TVC). When searching for specific and rather rarely occurring pathogens such as antibiotic resistant bacteria of if the water is of appropriate quality and therefore contains a low bacterial load, prior membrane filtration can be useful. For this purpose, a selected volume of the sample is filtered through a cellulose nitrate filter with a defined pore size. The relevant bacteria remain on the filter surface, which is then placed on a culture medium and incubated. The nutrients diffuse through the filter and thus enable bacterial growth (Pichhardt, 1998).

Dusts in livestock housing are composed of particles of litter, feed, feces, animal skin, pollen, fungal spores, insect parts, dead and living microorganisms and hair (Hoy et al., 2016). These dusts are often carriers of infectious agents, such as bacterial, viral, protozoal or fungal pathogens (Sommer et al., 1991; Dungan, 2010). The survival time of airborne microorganisms

depends on the ambient temperature and humidity, whereas spores, as resistant permanent forms, can persist for a long time even under adverse conditions. A high dust load in the stable can also lead to mechanical or toxic damage of the lung tissue, especially if the particles are very small (< 5 µm) and therefore alveolar (Hoy et al., 2016). The dust content and also the germ content in the stable air depend on the animal species, the type of housing and especially the use of litter. For example, the average content of aerobic bacteria in the air in stables varies from 85 cfu per liter air for calf housing to 5,000 cfu per liter air for chickens in floor housing (Müller et al., 2011). The sedimentation time depends on the particle size. Very small particles, i.e. fine dust (< 5 µm), remain dispersed in the air, whereas particles larger than 100 µm sediment relatively quickly on surfaces. Dust particles whose sizes are between (5 - 100 µm) can settle on contaminated surfaces and take up pathogens or endotoxins even from areas inaccessible to animals. The contaminated dust particles then move again through air currents and deposit onto other surfaces, spreading the collected pathogens (Chauveaux, 2015).

For the examination of stable dust, dust can be collected directly from surfaces with a sterile brush. After successful cleaning and disinfection, however, there should not be enough dust remains on surfaces to be able to collect an appropriate amount with this method. Instead, the air in the stable can be sampled directly for microbial examination. There are different methods used to determine the airborne microbial count or to test for certain microorganisms. The simplest method is sedimentation, whereby culture media in petri dishes are simply placed open for a defined time at specific locations. The germs in the air deposit on the culture medium by sedimentation. The time in which the petri dishes are left open depends on the expected amount of bacteria (Pichhardt, 1998). Advantages of this method are the low personnel, technical and equipment requirements. Different culture media can be set up directly at the same time. During the sedimentation time, other work can be carried out. The disadvantage here is that only qualitative and not quantitative results are obtained. In addition, the whole measurement strongly depends on the environmental conditions and particle size (Juozaitis et al., 1994). In livestock housing, the measurement is significantly influenced by the distance to ventilation systems and possible draughts (Schaper, 2004). Quantitative information on the bacterial content in a defined volume of air is possible with the impaction method. For this method, a defined amount of air is suctioned through an air sampler. The air is then flowed directly onto the surface of an inserted solid culture medium, where the germs are separated from the air. Selective or non-selective culture media can be used, which are placed directly in the incubator afterwards. An advantage here is the simple application and the low expenditure of instruments.

The procedure is technically simple and can be carried out with little previous knowledge. Furthermore, no additional processing steps are necessary. A disadvantage, however, is the long time for sampling. Because if several bacteria species are to be determined, a new measurement with a new selective medium must be initiated for each species. In addition, in stables with a high dust content the dust particles deposit on the surface of the culture media, too, which can impair bacterial growth or impede colony counting.

A further quantitative possibility of airborne microorganism measurement is the impingement method. Here a defined amount of air is aspirated by the air sampler and directed into a liquid in which airborne particles such as dust, microorganisms, spores or pollen are collected. In the laboratory, the different species of bacteria can be examined directly from the same liquid sample. The advantage is that the bacterial content can be better determined by preparing necessary dilution steps, and species occurring in rather small quantities such as MRSA can also be examined in addition to the TVC (Juozaitis et al., 1994). Also for the determination of different species, only one measurement in the stable is needed. The disadvantage here is that the subsequent laboratory work requires more time. It is possible that a fraction of the bacteria is discharged from the air without being deposited in the liquid (Pichhardt, 1998), which can be reduced by adding a surfactant such as polysorbates to the solution to reduce the surface tension of the liquid.

Table 1.2 gives an overview of previous studies on the various hygiene indicators from healthcare sector, food industry and agriculture.

Table 1.2. Comparison of advantages and disadvantages of tested hygiene indicators, derived from several studies of the healthcare, the food industry and the animal production sector.

Reference	Sector	Research issue	Methods used	Size of the sampled area	Cut-off value	Advantages / Disadvantages
Lewis et al. (2008)	Healthcare	Assessing cleanliness	Visual inspection	Not specified	Not specified (tested for visual soiling, staining, foreign objects, surface condition, presence of moisture)	Not reliable
			ATP	100 cm^2 whenever possible	< 250 RLU (probably per area assessed)	Instant feedback; no information about remaining microorganisms
			ACP (TVC)	Not specified (probably limited by the size of the ACP)	< 2.5 cfu · cm^{-2}	No relation to pathogens and risk of infection
			ACP (*Enterobacteriaceae* and *S. aureus*)	Not specified	< 1 cfu · cm^{-2}	Pathogens difficult to isolate from environmental samples
Andersen et al. (2009)	Healthcare (patient rooms, not specified)	Comparison of different floor cleaning methods	ATP (Biotrace Int., Biotrace; Hygiena Int., Hygiena)	100 cm^2	Not specified	Easy to use; scale of interpretation varies between manufacturer
			ACP (TVC)	20 cm^2	Not specified	
			Air (TVC with impaction air sampler)	1,000 L	Not specified	
Sherlock et al. (2009)	Healthcare (Medical ward and surgical ward)	Assessing cleanliness	Visual inspection	Not specified	Not specified (absence of soiling, staining, presence of moisture)	Imprecise; subjective; inadequate; not sensitive
			ATP (3M, CleanTrace)	100 cm^2	< 500 RLU (probably per area assessed)	Fast; expensive; no correlation with TVC; useful for hygiene education programs or staff training
			Swabs (TVC)	100 cm^2	< 2.5 cfu · cm^{-2}	Precise; slow; no relation to pathogens and risk of infection
			Swabs (MRSA)	100 cm^2	Non detectable	Expensive

(Continuation) Reference	Sector	Research issue	Methods used	Size of the sampled area	Cut-off value	Advantages / Disadvantages
Mulvey et al. (2011)	Healthcare (Medical ward and surgical ward)	Assessing cleanliness and introduction of benchmarks	Visual inspection	Not specified	Assessed on a scale of 1 to 10	No correlation to ATP, TVC or *S. aureus;* not reliable
			ATP (Hygiena Int., Hygiena)	25 cm^2	100 RLU (probably per area assessed)	No relation to pathogens and risk of infection; combination of ATP and TVC to identify patient risk and unacceptable soil
			ACP (TVC)	25 cm^2	< 2.5 cfu · cm^{-2}	Relationship between microbial growth and ATP; no relation to pathogens and risk of infection
			ACP (*S. aureus*) (grown colonies additionally tested for methicillin-resistance)	25 cm^2	< 1 cfu · cm^{-2}	Good hygiene indicator in hospital settings to lower patient risk of infections
Alfa et al. (2013)	Healthcare (Laboratory simulation)	Validation of ATP to assess cleanliness of endoscopes	ATP (3M, CleanTrace)	Endoscopes were cleaned after artificial soiling. For sampling they were flushed with a defined volume (20 mL and 40 mL) of purified water Further tests were performed directly out of the flushing water	< 200 RLU	ATP correlated well with quantitative protein, hemoglobin and bioburden assay (Data not shown, statistical analysis not specified)
			Protein (photometric at 562 nm; similar to protein rapid test)		< 6.4 µg · cm^{-2}	Not concluded
			Hemoglobin (ELISA)		< 2.2 µg · cm^{-2}	Not concluded
			Enumeration of bacteria from artificial soil		< 4.0 log_{10} cfu · cm^{-2}	Not concluded

(Continuation) Reference	Sector	Research issue	Methods used	Size of the sampled area	Cut-off value	Advantages / Disadvantages
Lutz et al. (2013)	Healthcare (Laboratory simulation)	Comparison of methods for detection of *S. aureus*	ACP (*S. aureus*)	28 cm^2	Not necessary for comparison of methods	Performance of ACP and roller contact sampler depending on inoculating concentration; easy to use; time efficient; dirty surfaces result in debris and overloading; poor recovery at low bioburden
			Roller contact sampler (*S. aureus*)	100 cm^2		Better performance than ACP; sampling of large areas possible; easy to use; time efficient; dirty surfaces result in debris and overloading
			Rayon swabs (*S. aureus*)	36 cm^2		Well performance when corrected for area actually sampled; pre-enrichment possible
			Electrostatic wipes (*S. aureus*)	100 cm^2		Best performance across all inoculating concentrations; pre-enrichment possible; sampling of large or irregular areas possible
Mitchell et al. (2015)	Healthcare (Patient care area and general ward area)	Assessing cleanliness	Visual inspection	Not specified	‘Clean’ or ‘not clean’	Not reliable
			UV-Marker with fluorescent light assessment	Not specified	‘Clean’ (no fluorescence visible) ‘not clean’ (fluorescence still visible)	More objective; improved cleaning process
Casini et al. (2018)	Healthcare (diabetology ward and wound care ward)	Assessing cleanliness	ATP (3M, CleanTrace)	100 cm^2	40 RLU · 100 cm^{-2} for medium-risk areas 50 RLU · 100 cm^{-2} for low risk areas	Simple to perform; quick response; interfering with disinfectants possible; helpful to monitor cleaning quality
			Swabs (TVC)	100 cm^2	100 cfu · 100 cm^{-2} for medium-risk areas 250 cfu · 100 cm^{-2} for low risk areas	Microbial sampling necessary to gain reliable information on microbial dirt and patient risk
			Swabs (*S. aureus* and *Enterobacter* spp.)	100 cm^2	Not specified	
			Air (TVC by passive sedimentation)	1 hour	Not specified	Reflects risk of microbial wound contamination
			Air (TVC with impaction air sampler)	500 L	Not specified	Performed to obtain information on the concentration

(Continuation) Reference	Sector	Research issue	Methods used	Size of the sampled area	Cut-off value	Advantages / Disadvantages
Cunningham et al. (2011)	Food industry (Food Service)	Assessing cleanliness	Visual inspection	Not specified	Clean or not clean	Unreliable
			ATP (3M, CleanTrace)	100 cm^2	< 200 RLU Cutting boards and door handles: < 1,000 RLU	Rapid; easy to use; highly sensitive; slight positive correlation with ACP
			ACP (TVC)	50 cm^2	< 125 cfu · cm^{-2}	Expensive; time-consuming; inefficient in enumeration of microbes on surfaces
Osimani et al. (2014)	Food industry (Canteen)	Assessing cleanliness	ATP (3M, CleanTrace)	100 cm^2	Depending on cleanability of surfaces Easy to clean and smooth: < 100 RLU · 100 cm^{-2} Intermediate to clean: < 150 RLU · 100 cm^{-2} Porous materials: < 400 RLU · 100 cm^{-2}	Potential for real time monitoring; highly significant correlation between ATP and TVC; self-evaluation of the staff possible; ATP cannot substitute cultural methods
			Swabs (TVC)	100 cm^2	Clean ≤ 10 cfu · cm^{-2}	
			Swabs (*E. coli* and Coliforms)	100 cm^2	*E. coli* ≤ 1 cfu · cm^{-2} For coliforms not specified	
Öz & Arun (2019)	Food industry (laboratory simulation and poultry processing plant)	Comparison of ATP with cultural methods on artificial soiled stainless steel	ATP (SystemSURE Plus, Hygiena)	100 cm^2	clean < 10 RLU conditionally clean 11-29 RLU dirty > 30 RLU	Useful for monitoring cleaning performance on clean surfaces; correlation between ATP and cultural methods; ATP results depending on inoculating concentration (better performance at higher concentrations) and bacterial species (detection of *Salmonella* spp. better than *Saccharomyces cerevisiae*); low reliability on naturally contaminated surfaces; no information on bacterial species
			Swabs (TVC, *E. coli*, *S. aureus*, *Salmonella* spp.,	100 cm^2		

(Continuation) Reference	Sector	Research issue	Methods used	Size of the sampled area	Cut-off value	Advantages / Disadvantages
Vilar et al. (2008)	Animal production (Dairy)	Validation of ATP to assess cleanliness of milking equipment	ATP (Merck, HY-LYTE)	1 cm^2	152-1821 RLU depending on the sampling site	Fast; simple; well suited for field use; objective; useful to demonstrate effectiveness of cleaning to farmers; prediction of TVC of bulk tank milk by ATP values from milking equipment samples is not possible
			TVC in bulk tank milk	Not specified	Differentiation in three classes: A < 20 · 10^3 cfu · ml^{-1} B < 20-100 · 10^3 cfu · ml^{-1} C > 100 · 10^3 cfu · ml^{-1}	
Huneau-Salaün et al. (2010)	Animal production (Laying hens)	Assessing cleanliness	Visible inspection	Not specified	0 = dirty 1 = not completely cleaned, traces of dust, feathers, egg or manure 2 = clean	Useful for first impression; unreliable; no correlation with ACP
			ACP (*Streptococci* spp.)	25 cm^2	Not specified	Only useful on perfectly (visibly) clean surfaces; cannot be interpreted if macroscopic particles have stuck on the surface; useful to reveal critical points in sanitation
Luyckx et al. (2015)	Animal production (Fattening chicken)	Comparison of methods to assess cleanliness	Visual inspection	Not specified	Not necessary for comparison of methods	Not reliable
			ATP (Hygiena)	100 cm^2		Objective compared to visual inspection
			ACP (TVC, *E. coli* *Enterococci* spp.)	25 cm^2		Easy to process; fast to apply; sampling only of small areas possible; sometimes unreadable or overgrown
			Swabs (TVC, *E. coli* *Enterococci* spp., qualitatively: *Salmonella* spp.)	625 cm^2		Useful for sampling irregular or larger surfaces More handling and processing

(Continuation) Reference	Sector	Research issue	Methods used	Size of the sampled area	Cut-off value	Advantages / Disadvantages
Clemensson Lindell et al. (2018)	Animal production (Dairy)	Validation of ATP to assess cleanliness of milking equipment	ATP (3M, CleanTrace)	Different sizes: 16.5-80 cm^2	Clean: ≤ 150 RLU Acceptable, but indicating deteriorating: 151-299 RLU Dirty: ≥ 300 RLU	Strong correlation between ATP and TVC Not influenced by testing of wet equipment Replicate sampling over time necessary for correct interpretation If ATP values are very high, additional microbial testing is recommended for verification
			ATP in water samples (3M, CleanTrace)		Good hygiene: < 100 RLU Poor hygiene: > 200 RLU	
			ACP (TVC)	Not specified	Improperly cleaned: 45 cfu · 10 cm^{-2}	
			Swabs	10 cm^2	5 cfu · cm^{-2}	

1.4. Derived research questions and outline of the thesis

From the literature research it became clear that the topic of hygiene management, with cleaning and disinfection as integral parts is still highly relevant, even if the benefits have been known for a long time. Measuring systems already exists, which are regularly used to indicate the success of hygiene in the healthcare sector and in the food industry. Occasionally, there are also studies in which these hygiene indicators have been tested in animal production. However, a comparison of the advantages and disadvantages of the different systems for use in animal production is still missing. As a consequence, the first two research questions were derived:

- Which hygiene indicators are suitable for use in animal production?
- What are possible influencing or disturbing variables, which have to be considered for successful implementation of hygiene indicators?

To assess the thoroughness and success of cleaning and disinfection procedures and to improve hygiene management concepts, it is important to know possible weak and critical points in order to be able to work systematically on their improvement. This leads to the next research question:

- What are critical points in routine cleaning and disinfection in farm animal housing?

Finally, the collected results of the examinations and the many experiences acquired during the farm visits lead to the last research question:

- How can farmers use the scientific findings to improve hygiene management in practice?

These research questions will be answered in the published studies presented in the next three chapters and will be further debated in the concluding discussion.

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2. Individual training for farmers based on results from protein and ATP rapid tests and microbiological conventional cultural methods improves hygiene in pig fattening farms

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2.1. Abstract

Optimal hygiene management is an essential part of maintaining a high standard of health in conventional pig production systems and for the successful interruption of infection chains. Currently, efficiency assessments on cleaning and disinfection are only performed by visual inspection or are neglected completely. The aim of this study was to evaluate the available methods for on-farm-monitoring of hygiene, identify critical points in pig pens and use the data obtained for training purposes. In addition to visual inspection by assessing the cleanliness, microbiological swab samples, i.e., aerobic total viable count (TVC), swab samples for adenosine triphosphate (ATP) as well as protein residues and agar contact plates (ACP) combined with three different culture media, were applied and ranked according to their suitability for livestock farming. Samples were collected on at least fifteen critical points from one representative pen on six pig fattening farms with various hygiene management practices after cleaning and disinfection. After the first sampling, farmers were trained with their individual results, and sampling was repeated six months after training. Nipple drinkers, feeding tubes (external and inner surface) and troughs (external and inner surface) showed the greatest bacterial loads (TVC: 4.5 – 6.7 $\log_{10}$ cfu $\cdot$ cm^{-2}) and values for ATP and protein residues; therefore, these surfaces could be identified as the most important critical points. Spearman rank correlations ($P < 0.01$) were found between the different assessment methods, especially for the TVC and ATP ($r = 0.82$, $P < 0.001$). For rapid assessment on farms, ATP tests represented an accurate and cost-efficient alternative to microbiological techniques. Training improved cleaning performance as indicated by a lower rating for visual inspection, TVC, ATP, two categories of antibiotic resistant bacteria (methicillin-resistant *Staphylococcus aureus* (MRSA) and extended-spectrum β-lactamases producing bacteria (ESBL)) in the second assessment. The monitoring of cleaning efficiency in pig pens followed by training of the staff constitutes a valuable strategy to limit the spread of infectious diseases and antibiotic-resistant bacteria. Special attention should be paid to the sufficient hygiene of drinkers and feeders.

Key words: animal health, cleaning, disease prevention, disinfection, method evaluation, pork production

2.2. Introduction

Cleaning and disinfection of pens is an integral part of health management in livestock farming. The German law prescribes that pens and equipment for pig farming must be cleaned and disinfected between the housing out and restocking of animals (SchHaltHygV, 1999). However, methods for how sanitation could be monitored systematically are lacking. The most common method, visual inspection, depends on subjective perception and structural conditions such as light intensity or the color of surfaces. To make matters worse, not every soiling or bacterial contamination is visually perceptible; therefore, overestimation of cleanliness is likely (Sherlock et al., 2009). Furthermore, remaining organic material can significantly reduce the effect of applied disinfectants (Ward et al., 2006), which means that the efficacy of disinfection highly depends on the precision of the initial cleaning. How cleaning and disinfection should be carried out is mostly known; however, in practice, thoroughness often suffers from a lack of time. For training and consultancy purposes, easily understandable arguments can help to convince farmers to change their procedure. Changes lead to healthier animals and improve economic factors such as feed efficacy or therapy costs (Banhazi and Santhanam, 2013, Le Floc´h et al., 2014). In addition, decreasing the use of antibiotics reduces the development and spread of livestock-associated antibiotic-resistant bacteria (Gleeson and Collins, 2015). The first objective of this study was to find an appropriate method for assessing hygienic conditions in all-in all-out pig fattening systems. Therefore, different techniques, commonly used in hospital hygiene and food production, namely microbiological swabs, protein- and adenosine triphosphate (ATP) - rapid tests and different agar contact plates (ACP) were compared. The second objective was to suggest critical points in a pen suitable for routine monitoring after cleaning and disinfection. The third objective was to determine if training and raising awareness of the farmers by identifying individual hygienic condition results and critical points help to improve hygiene status and reduce exposure to pathogenic bacteria. The long-term objective is to enable farmers to develop farm specific solutions and continue improvements in hygiene.

2.3. Materials and methods

This study was conducted in accordance with federal and institutional animal use guidelines (Az. 84 - 02.05.40.16.038), data privacy agreement (University of Bonn, 38/2018) and ethical standards.

2.3.1. Selection of the farms and experimental design

For the study, six conventional pig fattening farms, located in northern Germany, with major differences in cleaning and disinfection procedures were chosen. Differences in hygiene management were assessed by a questionnaire before sample collection, containing information about used detergents and disinfectants as well as drying and exposure times. Two samplings were performed on each farm. The purpose of the first was to focus on suitable monitoring methods and identify possible control points. After data collection and processing, individual results were discussed with the farmers, and measures for improving the hygienic conditions were suggested. Sample collection was repeated six months after training to survey changes in hygiene management. The samples were taken on each farm after cleaning and disinfection immediately (0 to 24 h) before restocking. The farm hygiene protocol, especially cleaning and disinfection practices, varied depending on the farm-specific management (Table 2.1).

Table 2.1. Differences in hygiene practices of cleaning and disinfection procedures, and number of fattening places on pig fattening farms, depending on the farm.

	Use of detergents	Use of disinfection agents	Drying time before disinfection, hours	Exposure time to disinfectant agent, hours	Change of disinfectant agents	Type of production chain	Number of fattening places
Farm 1	No	Yes	4	16	Rarely	Integrated	1444
Farm 2	No	Yes	0,75	4	Rarely	Contracted	1250
Farm 3	Yes	No	-	-	-	Integrated	640
Farm 4	No	Yes	48	24	Rarely	Contracted	620
Farm 5	No	Yes	12	24	Always	Integrated	1120
Farm 6	Yes	Yes	6	6	Rarely	Contracted	3610

On each farm, 16 different sampling sites (Table 2.2) in randomly chosen pens were tested: entrance door (inside), back wall, side wall, ceiling, slatted floor, manure area, feeding area, feeding tube (upside), two nipple drinkers from the same pen, trough (outside), trough (inside), two manipulable materials (toys), window sill, and feeding tube (inside). Detailed information on the sampled areas and supplementary notes on the methods used are given in Table 2.2. Materials and surface roughness of the sampled areas were recorded (Supplemental Table 6.1, listed in the Annex). The different methods used and the specific purpose of the methods are given in Supplemental Table 6.2 (listed in the Annex). For the nipple drinkers, the inner nipple and the outer tube were swabbed in a circular motion. On planar surfaces, samples were taken

by wiping the area horizontally and vertically. For every sampling point, an area of 25 cm^2 was tested. Swabs were premoistened with sterile physiological saline solution (Oxoid, BR0053, Basingstoke, UK). Each farm was visited twice, so a total of 216 samples were taken from the six farms. All samples were stored in chilled insulated boxes (4 – 7 °C) and transported to the laboratory and examined within 24 h.

Table 2.2. Defined sampling points and possibility of sampling on pig fattening pens, which partially provide direct animal contact.

	Animal contact	Swabs[1]	ACP[2]	Sampled area, 25 cm^2
Entrance door (inside)	Yes	Yes	Yes	50 cm height
Back wall	Yes	Yes	Yes	50 cm height
Side wall	Yes	Yes	Yes	50 cm height
Ceiling	No	Yes	Yes	Middle of the pen
Slatted floor	Yes	Yes	Yes	Middle of the pen
Manure area	Yes	Yes	No	50 cm length, feces corner
Feeding area	Yes	Yes	Yes	10 cm in front of the trough
Feeding tube (upside)	No	Yes	Yes	Center above the pen
Nipple drinkers	Yes	Yes	No	Inner and outer tube
Trough (outside)	Restricted	Yes	Yes	Center, including the fold
Trough (inside)	Yes	Yes	Yes	Center, inner side wall
Manipulable material	Yes	Yes	Yes	Intensively used area
Window sill	No	Yes	Yes	Center
Feeding tube (inside)	No	Yes	No	Inner tube

[1]Includes all microbiological swabs (aerobic total viable count (TVC), total coliform count (TCC), MRSA and ESBL) and swabs for rapid tests for ATP and protein.
[2]Agar contact plates (ACP) were not applicable to all sampling sites because of their shape.

2.3.2. Visual inspection

Before sampling, the visual cleanliness of the area was assessed by at least two persons using a three-score grading system (1 = cleaning was satisfactory, no remaining soiling visible; 2 = cleaning was sufficient, minor soiling visible; 3 = cleaning was unsatisfactory, coarse soiling visible). The observer team consisted of six persons in total, who were trained before to gain comparable results.

2.3.3. Microbiological swab samples

For the microbiological analysis, samples were taken by using sterile moistened flocked swabs with 1 ml of liquid Amies medium (eSwab, Copan, Brescia, Italy). The swabs were well mixed for 30 s to dissolve bacteria quantitatively. From the Amies medium, serial dilution series (1:10) were prepared in sterile saline solution (Oxoid, Basingstoke, UK) with 1 % tryptone (VWR, Leuven, Belgium) to produce countable results. For aerobic total viable count (TVC), three dilution steps were plated with nonselective plate count agar (Merck, Darmstadt, Germany) by pour plating in a dual approach. Plates were stored for 72 h at 30°C under aerobic conditions.

After incubation, all visible colonies were counted as viable numbers of microorganisms, expressed in colony forming units (cfu) per cm^2 or ml, from plates containing a minimum of ten and a maximum of 300 colonies. All microbiological data were log transformed. Additionally, samples were investigated for the number of total coliforms by pour plating with selective Chromocult coliform agar (Merck). After incubation for 24 h at 37°C, all dark blue to salmon red colonies were counted as total coliform bacteria. For qualitative analysis of methicillin-resistant *Staphylococcus aureus* (MRSA) and extended-spectrum β-lactamase-producing bacteria (ESBL), selective CHROMagar plates (Mast Group, Reinfeld, Germany) were used. With sterile spatulas, 0.1 ml of the samples was spread on the agar surface without prior dilution. The MRSA plates were incubated for 24 h at 41°C and afterwards, for further increased pigmentation, for another 24 h at room temperature, ESBL plates for 24 h at 41°C. Pink colonies from MRSA plates were transferred to Columbia sheep blood agar (Mast Group) and incubated for 24 h at 37°C for confirmation. Light-gray colonies producing characteristic β-hemolysis were counted as resistant *S. aureus*. All colonies that grew on ESBL agar were considered a positive result for an ESBL builder strain without further identification of the species.

2.3.4. Water samples

Additionally, for microbiological analysis of livestock drinking water, water samples with a volume of 50 ml (stagnating water) were taken. Two samples with equal volume from one pen per farm were mixed to generate a pooled sample. Samples were processed analogously to microbiological swab samples.

2.3.5. Sock samples

The pens were tested with sock samples, as routinely used for Salmonella monitoring in broiler houses. Sock samples were taken by covering sterile rubber boots with a disposable cellulose hair net and walking 50 steps through the pen in a serpentine motion including the corners. Socks were transferred to 100 ml of sterile saline solution. After blending with a stomacher for 60 s, the saline solution was analyzed. Microbiological cultivation for enumeration of TVC were performed analogously to swab samples.

2.3.6. Adenosine triphosphate (ATP) rapid test

For the analysis of ATP content, sterile premoistened ATP swabs were used (CleanTrace Surface ATP Test Swab UXL100, 3M, Neuss, Germany). This test system is based on a bioluminescence reaction, with ATP as a cofactor. After swabbing the targeted area, the ATP test was activated by pushing down the stick handle to remove the membrane and starting the enzymatic reaction by combining all chemical solutions. After 10 s of shaking, the amount of emitted light was measured by a luminometer (NG III, 3M) in relative light units (RLU). The resulting values are displayed in $\log_{10}$ RLU per cm^2 or ml.

2.3.7. Protein rapid test

Samples were taken by special swabs for a protein rapid test (Clean Trace, 3M). This semiquantitative test system is based on a chemical reaction, resulting in a color change, which depends on the protein content. The test system was activated by pushing down the stick handle and gently shaking to mix the reaction solutions. The results could be obtained visually after 15 min. For a rapid interpretation of the measured protein content, the resulting color change was assessed by a defined five-score color scheme (1 = no change, 5 = strong change from green to violet).

2.3.8. Microbiological agar contact plates (ACP)

All flat surfaces (Table 2.2) were tested with ACP. Three different commercially available media were used: a nonselective plate count agar (PC) for enumeration of TVC, violet red bile dextrose agar (VRBD) for selective cultivation of *Enterobacteriaceae* and Dey Engley Agar (DE) for cultivation of bacteria after disinfection to neutralize disinfectant residues (HygieneChek, 49404R, 49417R, 49428R, Romerlabs, Butzbach, Germany). All ACP had a surface of 9 cm^2. Bacteria were transferred to the media by gently pressing the agar on the sampling surface. After incubation for 24 h at 30°C, all grown colonies were counted. Sampling with ACP was only used in the initial sampling before training of the staff.

2.3.9. Hygiene management training and raising awareness

After data analysis and processing of the results from the first trial, all farm managers were invited for hygiene management training. At least one person from each farm participated. The training started with an introductory lecture about basic protocols of hygiene management, the differences in cleaning and disinfection and biological foundations of microbiological contaminations in a 60 min oral presentation. Sampling points were introduced briefly, and

participants were asked to guess their own results (one feedback form per farm, guesses were matched with more than one participant per farm). The general weak points (as group means) for cleaning and disinfection were noted in a short talk (less than 10 min). Subsequently, the individual results were handed out to each farmer in privacy with the possibility to ask questions. To highlight the critical points, the results were presented in bar charts and traffic light-colored for better visualization. Additionally, photos to show soiling were handed out. Farmers were encouraged to compare their individual results voluntarily and suggested measures for improving the hygienic status in a chaired group discussion afterwards. Two years after the initial sampling, all farmers were asked again whether they had changed their hygiene protocols in the long-term consequence of the training.

2.3.10. Statistical data analysis

Descriptive statistics were performed with Excel 2016 (Microsoft, Redmond, WA) by calculating percentages. The Wilcoxon signed-rank test was used to estimate differences between sampling points within each sampling technique, as well as training effects considering the results from the first and second sampling. Correlations were revealed by the Spearman rank correlation procedure of SAS 9.4 (SAS Institute Inc., Cary, NC). The level of significance was set at $P < 0.05$, with $P < 0.01$ as highly significant and $P < 0.10$ as a tendency.

2.4. Results

2.4.1. Critical points in hygiene

To identify critical points for the cleaning and disinfection of pig fattening farms, the TVC results are presented (Table 2.3), which are considered to be the gold standard for assessing hygienic conditions. The greatest bacterial loads from TVC swab samples were found for the nipple drinkers, feeding tubes (upside and inside) and troughs (inside and outside) after the first trial. Entrance doors, back walls, side walls, slatted floors and manure areas showed the lowest bacterial loads after the first trial. The median values of total coliform counts fell below the detection limit, except for feeding area, nipple drinkers, troughs (outside) and feeding tube (inside), which indicates hygienic problems in these areas.

Table 2.3 Minimal, median and maximal values of TVC, TCC, ATP and protein residues from swab samples and from different ACP used of the sampled points.

		TVC[1,3], log_{10} cfu · cm^{-2}			TCC[2,3], log_{10} cfu · cm^{-2}			ATP[4] residues, log_{10} RLU · cm^{-2}			Protein residues (numeric, 1 to 5)		
Sample point		Min.	Median[5]	Max.	Min.	Median[5]	Max.	Min.	Median[5]	Max.	Min.	Median[5]	Max.
Entrance door, inside	(n = 6)	0.4	2.9^{bc}	4.1	0.6	0.6	3.9	0.4	1.3^{d}	1.6	1	2	5
Back wall	(n = 6)	0.8	2.2^{a-d}	4.1	0.6	0.6	1	0.9	1.2^{cd}	1.7	1	1	5
Side wall	(n = 6)	0.8	2.7^{c}	5.9	0.6	0.6	2.9	0.4	1.6^{d}	2.1	1	3	5
Ceiling	(n = 6)	1.8	3.5^{a-d}	5.1	0.6	0.6	3	1.4	1.5^{bcd}	2	1	3	5
Slatted floor	(n = 6)	1.1	3.4^{a-d}	4.2	0.6	0.6	2.8	1.4	1.8^{a-d}	3.4	1	1.5	4
Manure area	(n = 6)	0.6	3.2^{a-d}	4.9	0.6	0.6	1.6	0.9	1.7^{bed}	2.5	1	4	5
Feeding area	(n = 6)	0.8	3.8^{a-d}	5.1	0.6	1.1	4.1	1.1	1.9^{bed}	2.2	2	3.5	4
Feeding tube, upside	(n = 6)	0.6	5^{a-d}	6.8	0.6	2	2.8	0.5	2.3^{a-d}	3.3	3	4.5	5
Nipple drinker	(n = 6)	4.8	5.6^{ab}	6.3	0.6	1.1	4.1	1.7	2.7^{abc}	3.5	1	5	5
Nipple drinker	(n = 6)	3.9	5.7^{ab}	6.9	0.6	1.6	4.1	2.3	3^{ab}	3.6	4	4.5	5
Trough, outside	(n = 6)	2.7	4.8^{a-d}	6.8	0.6	1.1	4.1	1.4	2^{a-d}	3.8	1	4	5
Trough, inside	(n = 4)	1.1	5.3^{a-d}	6.5	0.6	0.6	4.2	1.5	2.5^{a-d}	2.7	2	4	5
Manipulable material 1	(n = 5)	2.2	3.3^{a-d}	5.7	0.6	0.6	3.8	1.2	1.9^{a-d}	2.4	1	2	5
Manipulable material 2	(n = 5)	1.7	4.3^{a-d}	6.6	0.6	0.6	4.1	1.1	1.8^{a-d}	3.2	1	2	4
Window sill	(n = 4)	2.8	3.1^{a-d}	4.1	0.6	0.6	2	1.6	1.9^{a-d}	3.9	3	4	5
Feeding tube inside	(n = 3)	5.9	6.1^{a}	8	2.7	4.1	4.1	2.6	3.6^{a}	4.2	3	5	5

[1]Aerobic total viable count (TVC) in colony forming units (cfu) per cm^2.

[2]Total coliform count (TCC).

[3]Lower detection limit for TVC and TCC = 0.6 log_{10} cfu · cm^{-2}.

[4]Adenosine triphosphate (ATP) in relative light units (RLU) per cm^2.

[5]Median values within a column followed by no common superscript show significant differences ($P < 0.05$).

Continuation of **Table 2.3**.

Sample point		TVC ACP[6], cfu · cm^{-2}			DE ACP[6,7], cfu · cm^{-2}			VRBD ACP[6,8], cfu · cm^{-2}		
		Min.	Median[5]	Max.	Min.	Median[5]	Max.	Min.	Median[5]	Max.
Entrance door, inside	(n = 6)	0.2	7.3	33.3	0.2	6.3	33.3	0	0.3	1.2
Back wall	(n = 6)	2.7	33.3	33.3	1.6	18	33.3	0	0.2	2.9
Side wall	(n = 6)	0.4	20.9	33.3	0.6	11.3	33.3	0	0	0.1
Ceiling	(n = 6)	0	22.2	33.3	0.1	5.6	33.3	0	0.1	0.3
Slatted floor	(n = 6)	1.6	22.2	33.3	0.3	21.8	33.3	0	0.4	1.2
Manure area	(n = 6)	0.4	9.3	33.3	8.3	22.2	33.3	0	0.2	33.3
Feeding area	(n = 6)	16.7	33.3	33.3	5.6	33.3	33.3	0.8	1	33.3
Feeding tube, upside	(n = 6)	11.1	33.3	33.3	16.7	33.3	33.3	0	0.1	0.3
Nipple drinker	(n = 6)	-	-	-	-	-	-	-	-	-
Nipple drinker	(n = 6)	-	-	-	-	-	-	-	-	-
Trough, outside	(n = 6)	13.9	25	33.3	16.7	27.8	33.3	0	0.5	33.3
Trough, inside	(n = 4)	5.1	19.2	33.3	5.9	19.6	33.3	0	0.5	1.1
Manipulable material 1	(n = 5)	22.2	33.3	33.3	22.2	33.3	33.3	0.1	0.1	0.1
Manipulable material 2	(n = 5)	33.3	33.3	33.3	33.3	33.3	33.3	5.6	19.4	33.3
Window sill	(n = 4)	0.3	33.3	33.3	22.2	33.3	33.3	0.1	0.4	1.7
Feeding tube inside	(n = 3)	-	-	-	-	-	-	-	-	-

[5]Median values within a column followed by no common superscript show significant differences ($P < 0.05$).

[6]Upper detection limit for Agar contact plates (ACP) = 33.3 cfu · cm^{-2}.

[7]Dey Engley Agar (DE).

[8]Violet red bile dextrose agar (VRBD).

MRSA were predominantly detected on the sampling points ceiling, manure area, nipple drinkers, trough (inside and outside), manipulable material and inside feeding tubes, with the lowest incidence on pen walls (Fig. 2.1). Positive findings for ESBL were detected in at least 40 % of the tested inner surfaces of feeding tubes, troughs and nipple drinkers. On window sills, no ESBL were detectable. For ATP and protein residues obtained results were similar to TVC from swab samples. Visual inspection resulted in highest scores for feeding tubes (inside), troughs (outside and inside) and manipulable materials (1 and 2) and window sills, divergently to the other methods. The ACP were not applicable for nipple drinkers and inner surfaces of feeding tubes and could only be used on flat surfaces. Comparable to TVC results from swab samples, highest loads for TVC ACP and DE ACP could be obtained for the upside surfaces of feeding tubes. In contrast to TVC from swab samples high bacterial loads were also found for feeding area, manipulable material (1 and 2), window sills and in case of TVC ACP for back walls. The results for VRBD ACP were generally low, except for window sills. All farms showed high values for the TVC in animal drinking water samples and varied between 4.5 to 6.1 $\log_{10}$ cfu · ml^{-1} in the first trial. Due to the dependence of cleaning status on the sampled areas, no correlations between roughness and cleaning status were calculated.

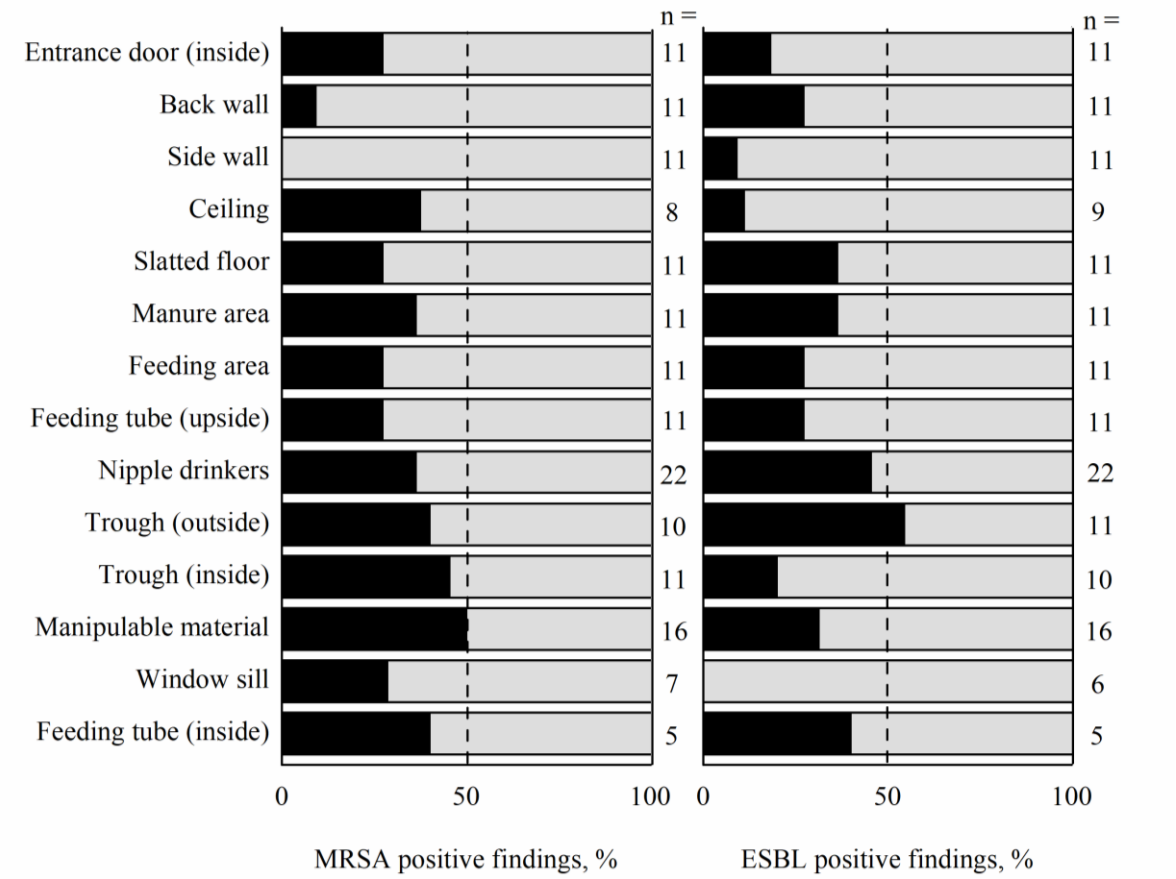
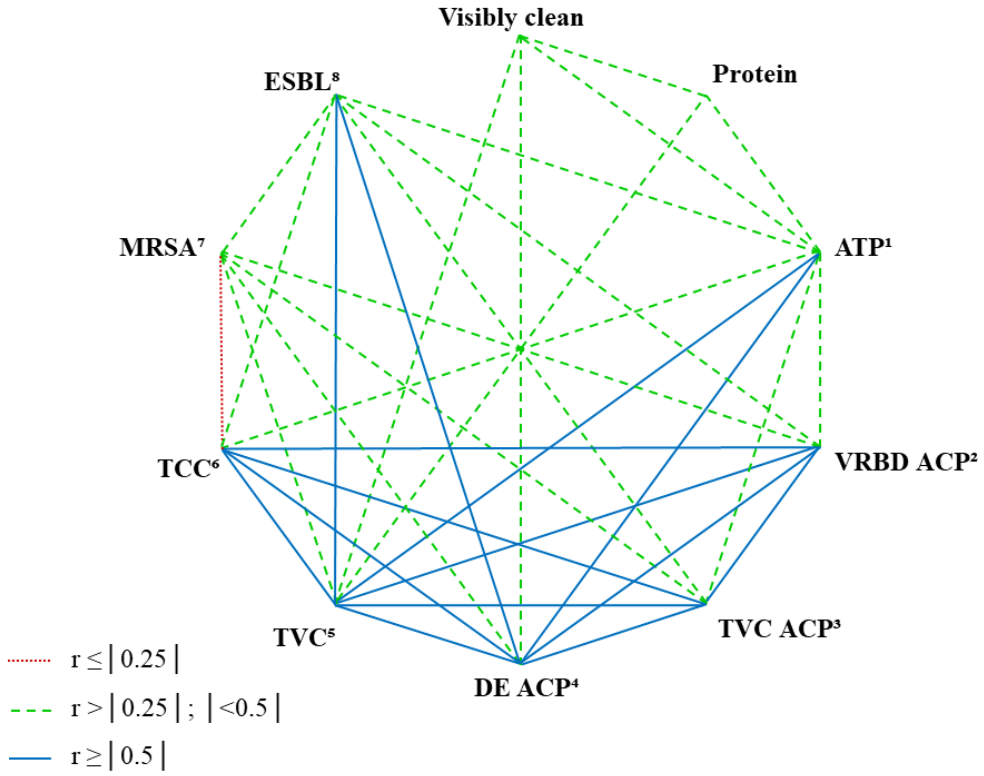


Figure 2.1. Percentage of positive findings of methicillin-resistant *Staphylococcus aureus* (MRSA) and extended-spectrum β-lactamase-producing bacteria (ESBL) in relation to the sampling points in pig stables (black = positive findings, gray = negative finding). Interpretation of the results should be considered with care, considering that the number of the samples varied between $5 \leq n \geq 22$.

2.4.2. Comparison of methods

After the first trial, correlations could be found between the different diagnostic methods (Fig. 2.2). Methods are compared with results from TVC of swab samples, which is the commonly used practice for evaluation of hygienic conditions. Aerobic total viable count correlated with results from protein tests, ATP residues, ACP, ESBL findings and visual inspection. For visual inspection, correlations with TVC, ATP, MRSA and ESBL were calculated. Evaluation of the surface roughness of the sample points resulted in correlations with the protein rapid test ($r = -0.31$, $P = 0.002$), TVC ACP ($r = 0.30$, $P < 0.02$), VRBD ACP ($r = 0.42$, $P = 0.002$) and DE ACP ($r = 0.29$, $P = 0.01$). Fifty-five percent of the TVC ACP ($n = 73$) and 48 % of the DE ACP ($n = 73$) were overgrown or unreadable due to dirt particles on the agar surface. For VRBD ACP, 8 % were unreadable ($n = 52$). For the total coliform count, 109 out of 168 samples were below the lower detection limit (2.0 $\log_{10}$ cfu · ml^{-1} and 0.6 $\log_{10}$ cfu · cm^{-2}, respectively) due to the sampling technique with swabs. Consequently, the total coliform count, as well as results from ACP were not further considered.



[1]Adenosine triphosphate with swabs
[2]Violet red bile dextrose agar with agar contact plates
[3]Aerobic total viable count with agar contact plates
[4]Dey Engley agar with agar contact plates
[5]Aerobic total viable count with swabs
[6]Total coliform count with swabs
[7]Methcillin-resistant *Staphylococcus aureus* with swabs
[8]Extended-spectrum β-lactamase-producing bacteria with swabs

Figure 2.2. Spearman rank correlations ($P \leq 0.05$) for the different techniques used to evaluate hygiene management on pig fattening farms at first sampling.

2.4.3. Training effect

To evaluate the effect of training and improved hygiene management, the results of the different sampling techniques from the first and the second trials were compared. The time point of examination shows highly significant correlations with ATP residues and the TVC of sock samples ($P < 0.01$), by comparing results from first and second sampling. Significant correlations were calculated for MRSA ($P < 0.02$) and ESBL ($P = 0.02$), depending on the sampling time. Most of the results from individual sampling locations for the TVC, ATP and protein residues showed a decrease in the second trial (Fig. 2.3). A significant training effect in form of a reduction for the single sampling locations between the first and second trial could only be obtained for the protein residues for one nipple drinker ($P < 0.01$) and a tendency for ATP residues ($P = 0.08$) for the nipple drinker. Another tendency to decrease was recorded for the TVC of the entrance door between the first and second sampling ($P = 0.08$).

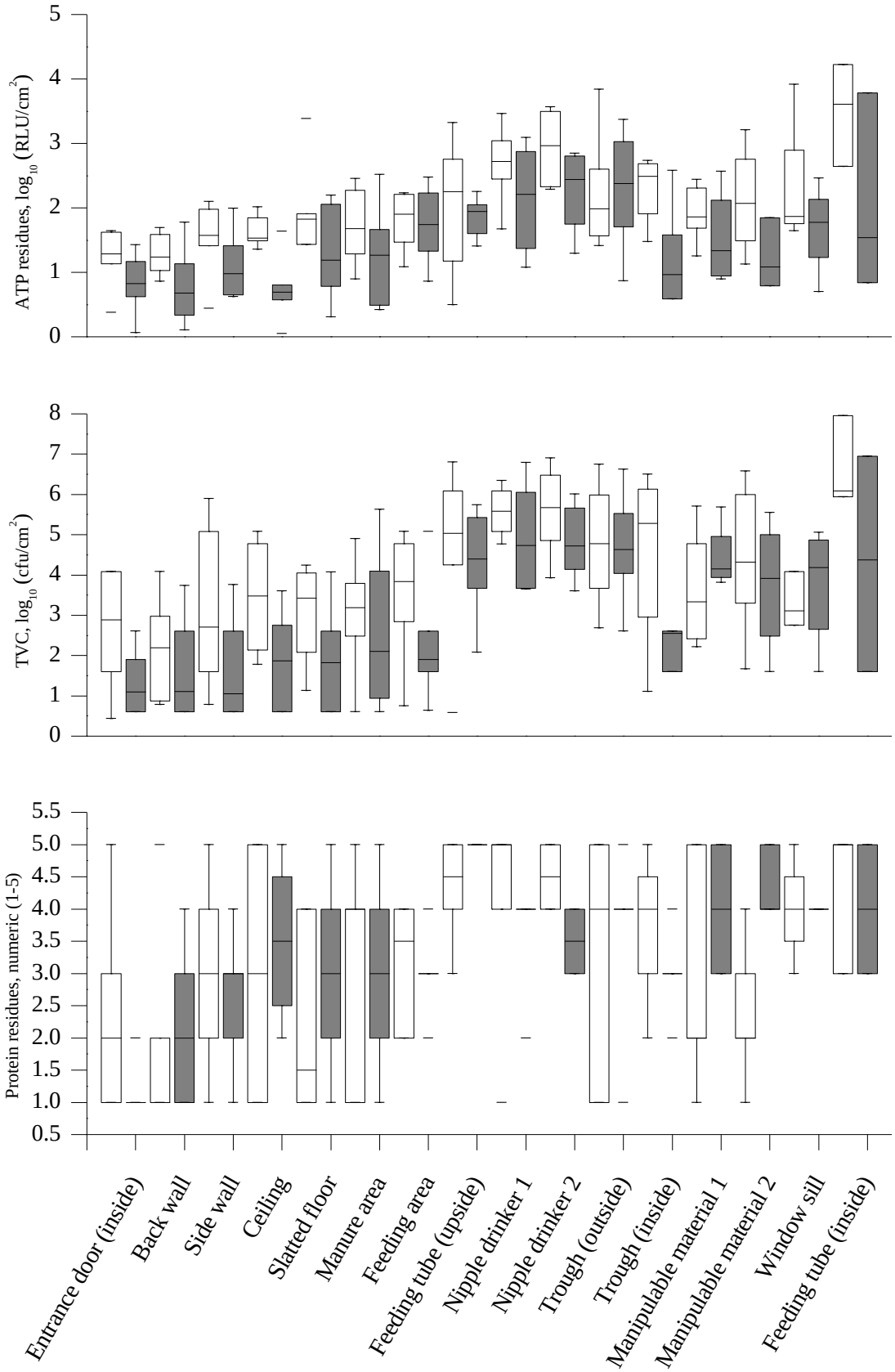


Figure 2.3. The effect of training the farmers of the pig fattening farms is shown by the reduction (time1: white boxes time 2: gray boxes) for adenosine triphosphate (ATP) (A), aerobic total viable count (TVC) (B) and protein values (C) for almost all sampled areas.

A tendency to decrease was found for the TVC of the swab samples in general ($P < 0.07$) and the TVC in water samples ($P = 0.06$) (Fig. 2.4). The TVC for sock samples decreased for all farms between the first and second trials ($P = 0.002$) (Fig. 2.4). The TVC for all water samples still exceeded the recommended value for the microbiological quality of drinking water but did not show a consistent trend. The findings for MRSA and ESBL decreased on all tested farms. Results for MRSA and ESBL from farm six were not analyzable. For three out of five farms, no MRSA could be detected in the second trial and for two out of five farms, no ESBL was detected in the second trial (Fig. 2.5). In the survey for long-term monitoring, farmers that attended the training improved their awareness of critical points for cleaning and disinfection and changed their hygiene management protocols. All farmers regularly point out their employees to farm specific weak points in cleaning. Four out of six farmers reported an extended time for high-pressure cleaning.

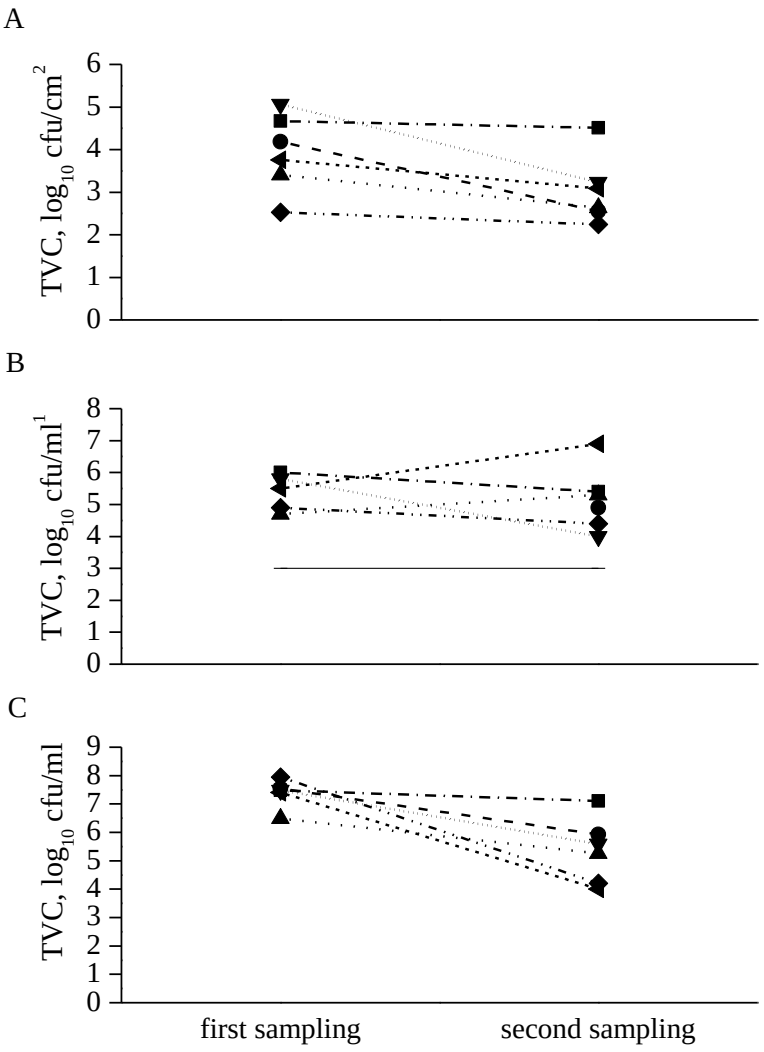


Figure 2.4. Development of aerobic total viable count (TVC) in swab samples (A), water samples (B) and sock samples (C) from six different pig fattening farms before and after training. On farm 2 no water sample was available during first sampling, because water mains were switched off.

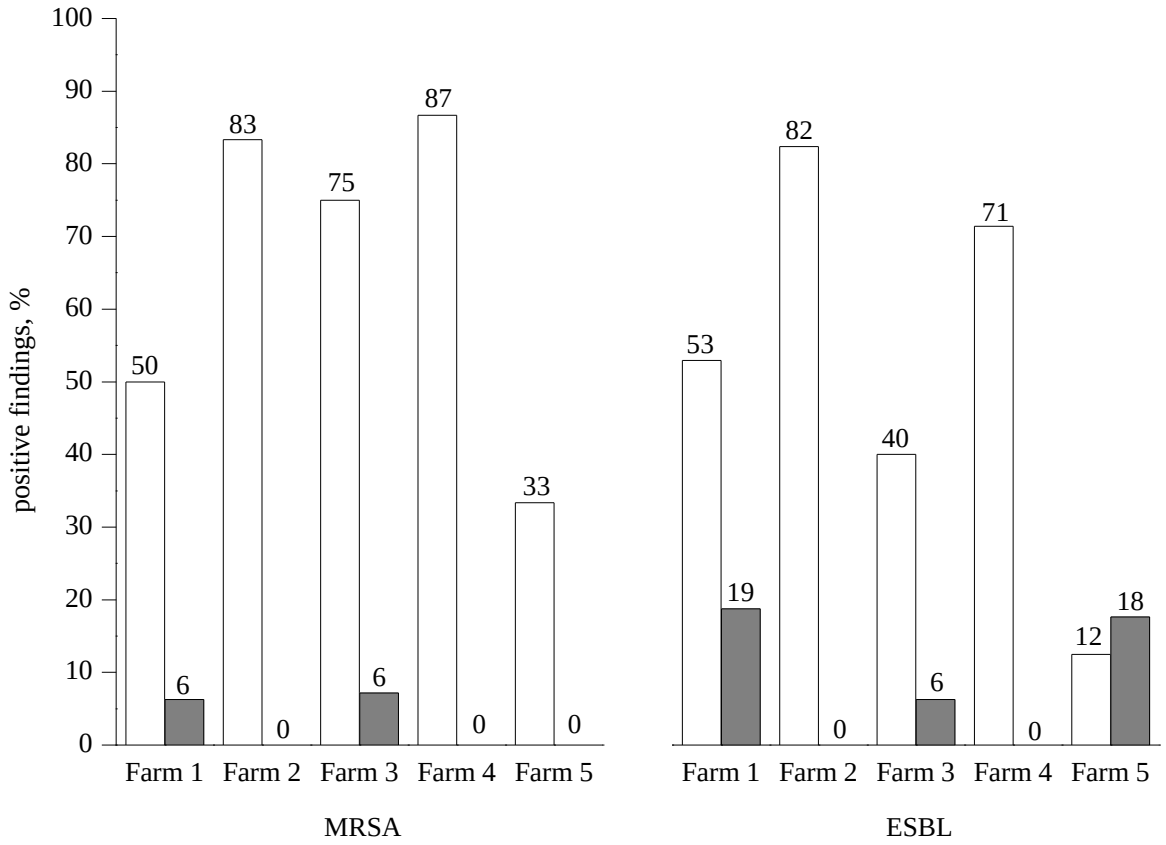


Figure 2.5. Percentage of positive findings for methicillin-resistant *Staphylococcus aureus* (MRSA) and extended-spectrum β-lactamase-producing bacteria (ESBL) after the first and the second sampling in relation to the pig fattening farms (time 1: white columns, time 2: gray columns). In the first sampling, samples for ESBL and MRSA from farm 6 were not analyzable.

2.5. Discussion

2.5.1. Hygienic critical points

The results show that nipple drinkers must be considered one of the most critical points in cleaning and disinfection procedures. This result was also found for nipple drinkers in pig nursery units (Luyckx et al., 2016). By measuring Enterobacteriaceae counts in pig finisher farms, Mannion et al. (2007) showed that feeders and drinkers are more contaminated after sanitation than floors, which is comparable to these results. They suggest that feeders and drinkers are resoiled during power-washing due to the splashing of contaminated water. Gonzalez et al. (2015) demonstrated in their study that the hygiene of feeders and drinkers is afflicted with problems, which could be confirmed in the present investigation. As a result of the insufficient cleaning of drinkers, the analyzed livestock drinking water samples were highly contaminated with bacteria. There is no legally determined upper limit for TVC in livestock drinking water in European law. In Germany, for the biological quality of animal drinking

water, a benchmark of 3.0 $\log_{10}$ cfu · ml^{-1} at 37°C or of 4.0 $\log_{10}$ cfu · ml^{-1} at 20°C is recommended by the Federal Ministry of Food and Agriculture (Kamphues et al., 2007), which was exceeded in almost all analyzed samples in the previous study. Pathogenic bacteria from animals of the previous batch can be easily transferred to newly arriving pigs via water intake from insufficiently cleaned drinkers. In a study that analyzed samples from six different pig farrow-to-finish farms with Rodac Plates, a special form of ACP, feeders and floors were critical points for cleaning and disinfection. In comparison, wall segments were fairly cleaned and disinfected (Vangroenweghe et al., 2009). The present study resulted in a particularly high contamination of feeders and a lower soiling of walls for TVC from swab samples. The results for the floor differ from those of Vangroenweghe et al. (2009), possibly due to the different sampling techniques. In general, sampling points that are not just in view and require bending down or looking up for visible inspection while cleaning and disinfection seem to be often forgotten. When interpreting the results, it should be considered that the sensitivity of the results is limited due to the small sampled area. It is obvious that a strict cleaning and disinfection protocol is necessary to maintain animal health; however, there are only very few studies available on this topic. The importance of effective cleaning and disinfecting as a substantial step to avoid the carryover of Salmonella in livestock has been demonstrated in several publications (Rose et al., 2000, Carrique-Mas et al., 2009, Gautam et al., 2013 Martelli et al., 2017). This effect can be applied to MRSA and ESBL, which could be considered indicator organisms for resistant bacteria in pig livestock production (Schmithausen et al., 2018). In livestock production, the occurrence of MRSA and ESBL depends, in addition to antibiotic usage, on the amount of dust and feces, and transmission via air to newly arriving pigs seems possible (Venglovský et al., 2011, Friese et al., 2012, Laube et al., 2013). Therefore, appropriate hygiene lowers the risk of colonization with resistant bacteria. In general, hygiene itself has already been suggested as a critical control point for on-farm assessment of pig livestock farms, with visual control at daily intervals (von-Borell et al., 2001). As a part of this, the control of cleaning and disinfection should be included. One possible suggestion is the implementation of so-called hygienogram scores, as already established in poultry farming, to improve routinely performed cleaning and disinfection (Vangroenweghe et al. 2009). In poultry production, hygienograms, which are generated by determining the TVC with ACP, are sampled by a veterinarian or an official body according to a determined protocol (Maertens et al., 2018). The integration of a system similar to hygienogram scores in piggery farm management could possibly improve cleanliness but needs to be further developed, especially considering that greatest bacterial loads were found at sampling points where ACP are not applicable. A

conceivable possibility would be the combination of a visual inspection and rapid tests to avoid the additional costs of a microbiological examination. Microbiological tests could then be used in cases of recurring health problems and severe illnesses.

2.5.2. Suitable measurement methods

Monitoring methods for sanitation and cleaning must be reliable and sensitive (Turner et al., 2010). For monitoring in livestock farming, excessive sensitiveness can be counterproductive because of the high bacterial load that remains, despite proper sanitation. For example, ACP seem less suitable for hygiene monitoring, even if they did correlate with TVC. Most of the ACP were overgrown, depending on the sampling location, or unreadable because of adhering dirt or dust particles and were therefore excluded from the second trial. Luyckx et al. (2015) reported similar results when using ACP in broiler houses. They found that ACP sampled from before cleaning were overgrown and noted that enumeration on ACP selective for *E. coli* allowed fewer countable results compared to enumeration of swab samples. Additionally, ACP are only usable on flat surfaces and are of limited use due to their fixed shape. Rapid tests for ATP and protein are very attractive for on-farm monitoring in contrast to microbiological swabs or ACP because of their short duration. Classical cultural analysis of remaining microorganisms is time consuming and requires high labor costs; in addition, depending on the analyzed bacterial species, the analysis can last up to 72 h. Usually, after this time, the new production cycle on fattening farms has already started, meaning it is too late for possible corrective actions. In this study, the results from ATP tests gave highly significant correlations with TVC, but users must be aware that ATP test reports represent more than just remaining microorganisms. Additionally, organic soiling from feed or feces may also lead to high ATP values, as ATP is an energy carrier in all prokaryotic and eukaryotic cells (Sherlock et al., 2009, Pistelok et al., 2016). The reliability of ATP tests depends on possible residues of cleaning or disinfecting agents, which can influence the results and lead to decreased or rarely decreased RLU values (Green et al., 1999, Turner et al., 2010). For routine use of rapid ATP tests, a pass or fail benchmark must be set by the user to allow a correct interpretation of the hygienic status. Other authors have specified that ATP tests have the advantage of more objective information than visual control (Luyckx et al., 2015), which also applies to the protein test; however, due to the costs per test, approximately 2.90 Euro, it is questionable whether farmers are willing to pay for performance monitoring. Other studies from the hospital sector have shown that visual feedback from rapid tests to the staff increases the thoroughness of cleaning (Goodman et al., 2008), which is possibly transferrable to routinely performed hygiene training for personnel in

animal production. Comparing ATP and protein rapid tests, ATP tests better reflect subtle differences than protein tests, in which only roughly different color graduations can be recognized visually.

2.5.3. Training effect

A training effect could be observed when comparing the results from the first trial with the second trial. The values for TVC, ATP and protein residues decreased numerical for almost all sampled areas. Especially for flat surfaces, such as walls, floors and the inner surface of the troughs, the TVC value dropped below 3 $\log_{10}$ cfu · cm^{-2}, which could be seen as a general target value in the prophylactic disinfection in animal houses, depending on the type of material (Böhm, 1998). However, the suitability of the target value is limited to sample points with a defined surface area and is not suitable for sock samples. To define a target value for sock samples, further investigations are needed. Only two out of six farmers in our study used detergent for cleaning, while the others cleaned the stables with water and high-pressure only. By training, farmers should be made aware that cleaning with detergents prepares stables optimally for subsequent disinfection (Hancox et al., 2013). To improve hygienic conditions on farms and enhance animal health, changes in the attitude toward management practices are fundamental (Becton, 2006, Gleeson and Collins, 2015). To convince farmers of the importance of proper hygiene management, persuasive arguments are needed, which should include not only economic aspects such as greater productivity but also the improvement of animal welfare (Pastorelli, 2012, Banhazi and Santhanam, 2013, Le Floc´h et al., 2014, Gosling, 2018). Time is a key factor that influences the thoroughness of cleaning and disinfection, which emphasizes the importance of knowledge of farm-specific weak points in sanitation (Gosling, 2014). A possibility for improving hygiene management could be the development of a farm-specific hygiene protocol in consultation with a supervising veterinarian; with this protocol, the individual work would be checked off by the individual carrying out the work, similar to already existing protocols in the food industry known as the control of self-monitoring. A regular in-house training, perhaps guided by a specific consultant and with a possible turnaround of once a year, represents a conceivable opportunity for improvement. Correct and successful cleaning and disinfection always rely on the proficiency of the person performing the work (Carrique-Mas et al., 2009, Martelli et al., 2017, Gosling, 2018). Targeted training with monitoring results can help to increase efficiency and prevent from becoming inattentive due to routine. An alternative for improving sanitary status lies in outsourcing to professional cleaning contractors, as is common in poultry production. In several studies, cleaning and disinfection performed by

professional cleaning companies was better than that by farm staff (Vangroenweghe et al., 2009, Maertens et al., 2018). Rapid tests may help farmers monitor the performance of professional cleaning companies. This question should be clarified in further studies. In conclusion, the awareness of the importance of hygiene in livestock production should be enhanced

2.6. References

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3. Hygiene management in newborn individually housed dairy calf rearing focusing on housing and feeding practices

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3.1. Interpretative summary

Hygiene management in newborn individually housed dairy calf rearing focusing on housing and feeding practices. By Heinemann et al. Hygiene is essential to avoid diseases in calf rearing but is often less considered in practice. To indicate weak points in the sanitation of feeding and housing equipment, different hygiene indicators were tested. Milk feeding buckets showed the highest bacterial loads after cleaning, especially when artificial teats were not removed for cleaning. The measured hygiene indicators differed greatly among farms, indicating the different perceptions of the importance of proper sanitation to interrupt infection chains and prevent health disorders.

3.2. Abstract

In calf rearing, the first weeks of life are critical and are associated with the highest mortality due to enteric and respiratory diseases. The aim of this study was to identify hygiene and management risk factors associated with feeding and housing equipment by tracing health-related indicators. On 11 farms, pens (N = 14) or hutches (N = 8) for individual calf rearing prepared for restocking and feeding buckets were visually scored for cleanliness and sampled with swabs (environment: N = 167; feeding equipment: N = 120). The sanitation of floors was tested with sock samples (N = 41). A total of 328 samples were analyzed for ATP and protein residues, aerobic total viable count (TVC), total coliform count (TCC), *Escherichia coli*, methicillin-resistant *Staphylococcus aureus* (MRSA), extended spectrum β-lactamase-producing bacteria (ESBL) and *Salmonella* spp. Bulk samples from the remaining soiling were collected and analyzed for *Cryptosporidium* in addition to rotavirus, coronavirus and *E. coli* K99 with a rapid immunochromatographic test. The highest bacterial loads (TVC, TCC and *E. coli*) were observed in feeding equipment, especially the inner teat of milk feeding buckets. Environmental samples, primarily the sidewalls and back walls of tested pens and hutches, exhibited the lowest bacterial counts and ATP and protein residues. Unscrewing the artificial teats from the buckets for cleaning increased the pass rates for ATP residues (odds ratio (OR) 2.20, 95% confidence interval (CI) 1.01 to 4.83) and TVC (OR 3.35, CI 1.30 to 8.60). The use of disinfectants was positively associated with visible cleanliness (OR 12.38, CI 4.67 to 32.81), low protein residues (OR 3.03, CI 1.51 to 6.07), TVC (OR 3.49, CI 1.69 to 7.22) and the absence of *E. coli* (OR 11.67, CI 1.35 to 100.98) in environmental samples from housing equipment. All samples were negative for MRSA, rotavirus and coronavirus. One bulk sample was positive for *Cryptosporidium*. In 10.5% of all samples, ESBL was detected, and in 6.8%, ESBL *E. coli* was detected, predominately in sock samples, followed by feeding equipment samples. In conclusion, there is still great potential to improve the implementation of hygiene measures in individual calf housing. In particular, more attention should be paid to the cleaning of feeding buckets and artificial teats, as this is a simple means of interrupting the possible spread of pathogens among calves.

Key words: suckling calf, health risks, hygiene, disease prevention

3.3. Introduction

Enteric diseases are still the most frequent reason for calf morbidity and mortality, with the highest risk in the first three weeks postpartum (Bendali et al., 1999; Svensson et al., 2006). Additionally, diarrhea can depress growth and development of calves and can cause considerable financial losses to commercial farms (de Graaf et al., 1999; Marcé et al., 2010; Torsein et al., 2011). Enteric infections with *Escherichia coli* K99, *Salmonella* spp., coronavirus, rotavirus or *Cryptosporidium* spp. require the frequent use of medicinal products. Good housing and hygiene management have the potential to decrease the incidence of diarrhea in young calves. Enteric diseases are usually transmitted via the fecal-oral route between excretors and recipients, and vertical transmission by housing equipment and horizontal transmission by feeding equipment are likely (Maunsell & Donovan, 2008). Infections caused by transmissions via surfaces hinge on different factors, including the load of pathogens, survival rate on surfaces, resistance to disinfectants and initial infecting dose (Tuladhar et al., 2012). Irregular or inadequate cleaning is one of the most common problems in calf-rearing, and preventive health interventions are sufficient tools for disease prevention by avoiding the transmission of infectious agents (de Graaf et al., 1999; Maunsell & Donovan, 2008; Barry et al., 2019a). The method and frequency of cleaning could significantly reduce the risk for diarrhea outbreaks in calves (Castro-Hermida et al., 2002; Klein-Jöbstl et al., 2014). In pork and poultry production, standardized methods for cleaning and disinfection are more common but still have weaknesses, such as inefficient cleaning of feeders and waterers or drain holes and floor cracks (Mannion et al., 2007; Mueller-Doblies et al., 2010; Luyckx et al., 2015). For calf-rearing, practical recommendations and knowledge about critical points in hygiene are hardly implemented in daily routine on farms, even though the importance of hygiene interventions is well known (Weaver et al., 2000; Barrington et al., 2002; Klein-Jöbstl et al., 2014). Additionally, although the EU legal requirements about hygiene in calf rearing state that housing pens for calves must be properly cleaned and disinfected (EU 2008/119/EC), appropriate time intervals, methods for documentation or procedures to evaluate success in sanitation remain unclear. The aim of this study was to assess management risk factors on dairy farms and gain information about weak points in the sanitation of newborn calf housing and feeding equipment by comparing different hygiene indicators to interrupt infection chains in the long term.

3.4. Materials and methods

This study was conducted in accordance with federal and institutional animal use guidelines (Az. 84 - 02.05.40.16.038), the data privacy agreement (University of Bonn, 38/2018) and ethical standards.

3.4.1. Selection of the farms

In consideration of the transport duration, a radius of 100 km around the laboratory was defined as the first selection criterion to ensure sample quality. A list of all dairy farms in this area was generated online at the website of the local chamber of agriculture. Based on the availability of phone numbers, 30 farms were contacted and asked about their willingness to participate, their work peaks and their number of cows. Eleven farmers refused participation directly. Emails with additional information about the overall study goals, planned tests, background information about the microbiological species, and time investment by the farmer were sent. Twelve farms out of 19 were preselected to cover variations between different farm sizes in the number of lactating cows representing variations in income, number of employees and routine in calf handling. One farm was discarded after the first visit because they changed their calf rearing system from individual housing to free mother-bonded rearing, so neither pens nor hutches were used henceforward. Ultimately, 11 dairy farms, varying between 60 to 700 Holstein-Friesian dairy cows and 60 to 800 calves per year, were chosen. All farms are individual- or family-owned companies, located in North-Rhine Westphalia, Germany. In Germany, newborn dairy calves are commonly separated from their dams immediately, fed with colostrum and housed individually for the first 14 days in all-in-all-out systems using either pens or huts until males are sold and females are kept in groups for restocking. During the first 14 days, newborn calves are fed milk diets (milk replacer, waste milk or bulk milk) by feeding buckets with artificial teats. The farm visits, including the interview, the visual inspection of the sampling points and further sampling, were always carried out by the same responsible person, accompanied by one assisting person, and both were experienced in sampling for hygiene measurements. Farm characteristics of the sampled farms were assessed by face-to-face interviews with the farm manager or the herd manager using an interview sheet, which was completed in compliance with the farm or herd manager, and an additional record sheet, which was completed in the absence of the manager (listed in the Annex). The in-house-developed interview sheet contained 67 closed-ended and semi-closed-ended questions dealing with hygiene management, feeding management and health-associated factors of individually housed calves. The interview sheet and record sheet were developed in consultation with two

experts in dairy calf management and were tested for understanding and plausibility in a preliminary trial with three farmers. Based on the results of the preliminary trial, the data sheets were revised. Sampling was performed at two different times on each farm. The objectives of the first sampling were to identify critical points in individual calf housing and to assess different hygiene indicators for their suitability in livestock farming. Sample processing was performed according to Heinemann et al. (2020). After processing the samples and data analysis, the results were discussed with the farmers. Sample collection was repeated after 305 ± 24 days to verify enhancements in hygiene. The ambient temperature during sampling varied between 4°C and 24°C, with a median value of 14.5°C. Data were checked for thermal effects, and since there were no significant effects, this was not included in any further model.

3.4.2. Assessment of housing situation and visible inspection

First, information about individual calf housing was recorded, e.g., pens or huts, dimensions, condition of the floor surface, and slope of the floor. Farm-specific characteristics were captured by photography. Visual cleanliness of the sampled areas was always registered with a three-score grading system (1 = no remaining soiling visible, 2 = minor soiling visible, 3 = coarse soiling visible), always by the same sampling person.

3.4.3. Sample collection

Samples were collected after the sanitation of empty calf housing huts or pens or boxes and from feeding buckets before restocking. Sampling was scheduled in consultation with the farmers. Samples were taken at the following defined locations in two feeding buckets and two huts or pens on each farm: the inner bottom of feeding buckets, inner surface of artificial teats, outer surface of artificial teats, entrance of huts or pens, sidewall of huts or pens, back wall of huts or pens, and middle of the floor in huts or pens. The two pens or huts and the feeding buckets were selected in a formal random process by choosing one from the edge and one from the middle. Sample collection was performed by using different kinds of swabs (swabs from rapid tests for the detection of ATP and protein residues and swabs for microbiological analysis, see below) by wiping an area of 25 cm^2 horizontally and then vertically while rotating the swab. Additionally, sock sampling, also known as ‘boot sock sampling’, which is the recommended method in the EU according to CR (EU) 200/2010 for detection of Salmonella in broiler houses and is routinely used for detection of *Salmonella* spp. and other bacteria, was performed (Skov et al., 1999; Berghaus et al., 2013). Compared with swabbing, boot sock sampling has the advantage of sampling larger areas of floor. For this purpose, clean and disinfected boots were

covered with disposable cotton hairnets as an absorbent coating, and a defined distance of 50 steps was walked through the sampled pen or hut. Care was taken to ensure that equal numbers of steps were always taken along the sides (20 steps) and diagonally through the pen or hut (30 steps). The hairnets were then transferred to sterile polyethylene bottles with screw caps in 100 mL of sterile saline solution for transport to the laboratory. *Cryptosporidium* spp., rotavirus and coronavirus were analyzed from a bulk sample. For this purpose, a sterile metal spatula was used to collect a minimum of approximately 10 g of remaining visible soiling from scratches and cracks in the floor or dried smears adhering to the walls or grids of the sampled hut or pen. All samples were stored in chilled boxes (4 to 8°C), transported to the microbiological laboratory (Biosafety level2) of the Institute of Animal Sciences of the University of Bonn and processed within 24 h. In total, 328 samples were analyzed.

3.4.4. Rapid tests

Two different kinds of rapid tests were used, which are routinely applied in the control of hygiene procedures of healthcare institutions and in the food industry: one to measure ATP residues (CleanTrace Surface ATP Test Swab UXL100, 3M, Neuss, Germany), which indicates soiling from feed, excrement or directly from bacteria, as ATP is the main energy carrier in all cells from plants, animals and microorganisms, and another to measure protein residues (Clean Trace Surface Protein Plus, 3M) originating from excrement and feed. Both tests were analyzed directly after sample collection on the farm. The ATP test emits light after an enzymatic reaction of the chemical compounds in the test system with ATP in the sample. The amount of light is proportional to the amount of ATP residue and is measured by a luminometer (NG III, 3M). The values obtained are given in relative light units (RLU) per mL. The protein test is a semiquantitative system in which a color change occurs, and the change depends on the amount of protein residue. The displayed color was assessed by a defined 5-score scheme (1 =no change, 5 = strong change).

3.4.5. Microbiological tests

Samples for microbiological tests were collected with sterile moistened flocked swabs with 1 mL of Amies medium (eSwab, Copan, Brescia, Italy). An adequate volume of Amies medium was diluted in physiological saline solution (Oxoid, Basingstoke, UK), depending on the expected amount of bacteria. Hair nets from sock samples and the 100 mL of saline solution into which the hair nets were placed for transport were mixed in filter bags with a stomacher to thoroughly dissolve the samples. The saline solution was processed in a manner similar to the

swab samples. All samples were investigated for aerobic total viable count (TVC), total coliform count (TCC), and *E. coli* by pour plating. Nonselective plate count agar (Merck, Darmstadt, Germany) was used for TVC, and Chromocult coliform agar (Merck) was used for the enumeration of *E. coli* and coliform bacteria. TCC and *E. coli* were used as indicators of fecal contamination. After incubation, the bacteria were counted, and the arithmetic mean was calculated and log transformed. The results are expressed as $\log_{10}$ cfu · mL^{-1}. Methicillin-resistant *Staphylococcus aureus* (MRSA) and extended spectrum β-lactamase-producing bacteria (ESBL) were cultivated using the spread-plate technique with selective CHROMAgar plates (Mast Group, Reinfeld, Germany). The Amies medium of the swab samples was directly pipetted onto the agar without prior dilution. To avoid excessive growth of accompanying environmental bacteria, plates were incubated at 41°C for 24 h. To distinguish blue colonies (potentially *Klebsiella* spp. *Enterobacter* spp. or *Citrobacter* spp.) from ESBL agar, the colonies were transferred to Columbia sheep blood agar (Mast Group), and further species identification was performed based on typical biochemical reactions with an EnteroPluri test (Liofilchem, Roseto degli Abruzzi, Italy). Pink to purple colonies were classified as ESBL *E. coli*. White colonies, which are *Acinetobacter* spp. or *Pseudomonas* spp., were not further distinguished due to their low pathogenic relevance in bovines. For pre-enrichment of *Salmonella* spp., the swabs were transferred to peptone water (Merck, Darmstadt, Germany) and incubated (24 h at 37°C). Afterwards, 1 mL of the pre-enrichment broth was added to 9 mL of Müller Kauffmann tetrathionate broth (BD Diagnostic systems, Heidelberg, Germany) and incubated for another 24 h at 37°C. Additionally, 0.1 mL of the pre-enrichment was added to 10 mL of Rappaport-Vassiliadis broth (BD Diagnostic Systems, Heidelberg, Germany) and incubated for 24 h at 41.5°C. Subcultivation of both broths was performed on xylose lysine deoxycholate agar (Merck, Darmstadt, Germany) and mannitol lysine crystal violet brilliant green agar (Merck, Darmstadt, Germany) on two consecutive days (incubation: 24 h at 37°C).

3.4.6. Detection of typical diarrhea-causing pathogens

The detection of *Cryptosporidium* spp. was performed microscopically and immuno-chromatographically. For microscopy, the collected bulk sample was processed according to the flotation method (Dryden et al., 2005). An amount of 5 g of the bulk sample, which mainly consisted of dried visible feces, was mixed into a saturated saline solution and filled in a glass cylinder until a slight positive meniscus was formed. A cover glass was placed on the top to allow the oocysts to float to the top and adhere to the cover glass. After 10 min, the cover glass was placed on a microscope slide and analyzed for oocysts of *Cryptosporidium* spp. with a

transmitted light microscope. Additionally, a rapid immunochromatographic test was used to simultaneously detect *Cryptosporidium* spp., rotavirus, coronavirus and *E. coli* K99 (Fassisi, Göttingen, Germany). This test is designed for the detection of antigens in the feces of calves. The sample material was dissolved in 5 mL of sterilized purified water and mixed with the reaction solution. Three to four drops were pipetted on the rapid test system according to the manufacturer's guidelines. After a reaction time of 10 min, the test was evaluated in binary categories. This test is actually designed for the examination of fresh calf feces. According to a literature search, it has not yet been used to measure reconstituted dried feces or aged feces; therefore, we cannot make any statement about the validity of our results. Thus, the results of this test should be interpreted with care and be considered as additional information to the main focus of this study.

3.4.7. Data analysis

All data from the record and interview sheet and from laboratory analysis were coded as numbers and summarized in an Excel file (Excel 2016, Microsoft Corp., Redmond, WA). Metric data were checked for normal distribution and log transformed for cfu from microbiologic tests and RLU from ATP tests. Data were analyzed using SAS 9.4 (SAS Institute Inc., Cary, NC). Based on major differences in cleaning frequency and the number of samples, data regarding hygiene indicators were grouped into 'feeding equipment' (results from the inner bottom of feeding buckets and the inner and outer surfaces of artificial teats), 'environment' (results from entrance, sidewalls, back walls, and floors) and 'sock samples'. The effects of sampling location within each hygiene indicator were tested with a general linear model with grouped location as the main effect (Fig. 3.3). Data from individual hygiene indicators were analyzed by the mixed model procedure with time, farm and time × farm interaction as fixed effects and sample as a random effect (Fig. 3.4 and 3.5). Differences were localized by Tukey's t test. Correlations between the different hygiene indicators were determined by the Spearman rank correlation procedure (Fig. 3.1 and 3.2). The results were considered significant at $P < 0.05$, with $P < 0.01$ indicating that the results were highly significant and $P < 0.10$ indicating a tendency. A generalized linear mixed model with dichotomized data (PROC GLIMMIX) was used for modeling the effects of feeding and housing management practices on hygiene indicators. Data were dichotomized using the following thresholds: according to Böhm (1998), 3.0 $\log_{10}$ cfu · cm^{-2} bacteria remain on surfaces in animal houses under practical conditions, even after sufficient cleaning and disinfection. This value, which corresponds to 4.4 $\log_{10}$ cfu · mL^{-1} in our study design, was set as a threshold for TVC. This approach was not

transferable to the sock samples due to the different sampling techniques. For sock samples, a TVC threshold of 5.5 $\log_{10}$ cfu · mL^{-1} was defined based on previous results (Heinemann et al., 2020). This value for the TVC of sock samples is considered to be achievable under practical conditions with sufficient hygiene management. The TCC and *E. coli* thresholds were set at the detection limit (feeding equipment and environmental samples: 2.0 $\log_{10}$ cfu · mL^{-1}, sock samples: 1.0 $\log_{10}$ cfu · mL^{-1}). MRSA and ESBL thresholds were based on the absence of susceptible colonies. The ATP threshold of environment samples and feeding equipment was adjusted based on the TVC threshold and set at 3.5 $\log_{10}$RLU · mL^{-1}. The protein threshold was set at a rating of 3 on the color scale.

3.5. Results

3.5.1. Characteristic farm management factors

The numbers of dairy calves born per year ranged from 60 to 800 animals. The median reported calf loss during the first 14 days was 0.8% (0 to 5.9%) (Table 3.1).

Table 3.1. Reported calf production data and rearing practices of the visited dairy farms sorted by number of calves per year.

Farm	No. calves per year	Losses during first 14 d	Losses during first 14 d, %	No. of rearing places	Contact between calves impossible	Calf huts	Calf pens
1	800	4	0.5	96	X		X
2	350	2	0.6	24			X
3	250	2	0.8	20		X	
4	200	5	2.5	23			X
5	130	1	0.8	16	X	X	
6	115	4	3.5	10		X	
7	110	1	0.1	15	X		X
8	110	0	0.0	14			X
9	85	5	5.6	5		X	
10	65	0	0.0	9			X
11	60	0	0.0	8	X		X

Generally, the farms showed substantial differences in management practices pertaining to housing and feeding (Table 3.1 and Fig. 3.1). The time at which the separation from the dam occurred varied between 1 and 24 h postpartum (median: 12 h). Newborn calves were kept in individual housing for the first 14 days on average (minimum at one farm: 10 days, maximum at two farms: 21 days). During the individual housing period, calves were housed in pens on seven farms (1.78 ± 0.29 m^2) and in huts on four farms (2.27 ± 0.13 m^2). All farms used straw as bedding material, and six renewed the bedding on a daily basis by adding clean straw on top

(one farm: every other day; three farms: only on demand; one farm: only at rehousing). Five farms housed newborn calves in brightly lit conditions instead of closed barns. All farms fed whole milk by feeding buckets, and seven additionally fed waste milk (milk from cows suffering from mastitis or being administered antibiotics) or milk with a high somatic cell count. Most commonly, milk was fed warm (10 of 11 farms) and nonacidified (7 of 11 farms). None of the farms used regular deworming agents. On seven farms, calves were treated with medicinal agents in the last six months (2x amoxicillin (β-lactam antibiotic), 2x halofuginone (coccidiostat), 1x metacam (analgesic), 1x treatment against acidosis, and 1x bromhexine (against respiratory disorders). Five of eleven farms reported diarrhea in calves at an incidence rate greater than 5%. Four out of these five farms with diarrheic problems commissioned a fecal analysis, which resulted in the detection of *Cryptosporidium* spp. and rotavirus or only *Cryptosporidium* spp. Three farms reported occasional respiratory disorders, and one farm reported a few umbilical infections. With regard to sanitation, all farms regularly cleaned the pens or boxes used for individual housing, but none of the farms used a fixed cleaning protocol. Cleaning was reported to be performed with pressure washers with water only (8 of 11 farms) and more rarely with the additional use of detergents (3 of 11 farms). Usually, farms routinely disinfect the pens after cleaning (7 of 11 farms) with disinfectants containing p-chlorocresol or combinations of glutaraldehyde, quaternary ammonium compounds and organic acids. All farms reported cleaning the buckets, but with substantial differences in the interval between cleanings. Four farms cleaned the buckets after every use, which meant twice a day, one farm cleaned the buckets weekly and the remaining farms cleaned them after each calf, which was equivalent to a 14-day interval. Cleaning methods differed between the farms and included cleaning with cold water only (7 farms), cleaning with cold water with detergents (2 farms), cleaning with hot water (1 farm) and cleaning with hot water with detergents (1 farm). The feeding bucket was disassembled for cleaning on six farms. Disinfecting the buckets was uncommon and was only performed on two farms. The reported diarrhea frequency correlated positively with disinfection of the pens after cleaning ($r_{Spearman} = 0.56$; $P < 0.001$) and waste milk feeding ($r_{Spearman} = 0.66$; $P < 0.001$, Fig. 3.1).

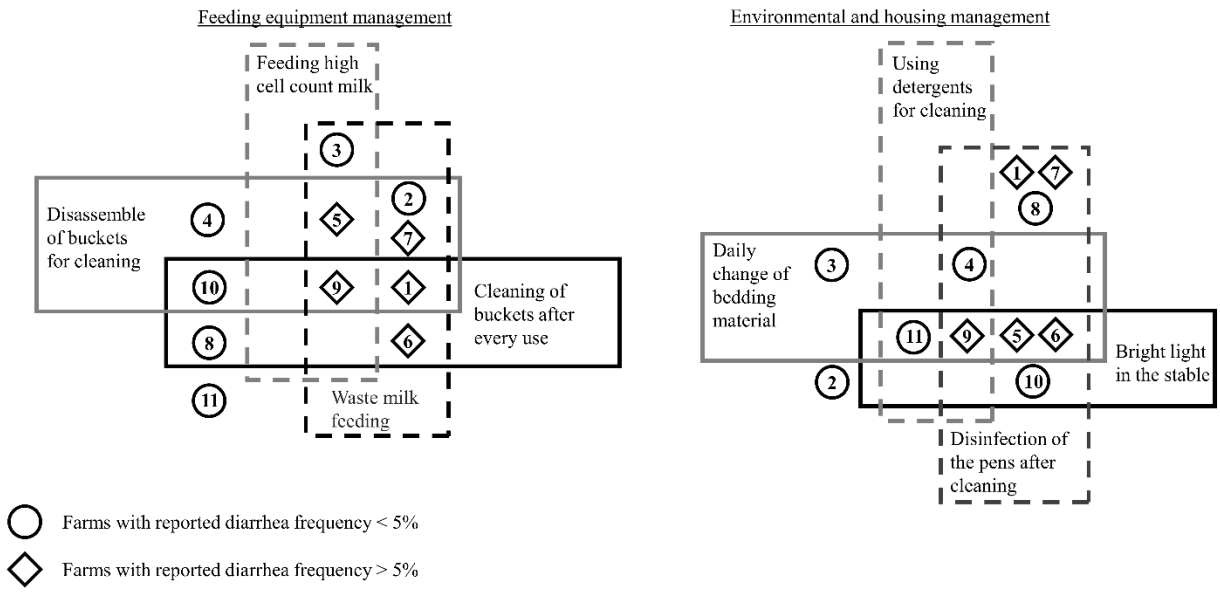

Figure 3.1. Comparison of different feeding and housing management practices depending on the reported diarrhea frequency of the farms.

3.5.2. Comparability of hygiene indicators

Spearman rank correlations between the applied hygiene indicators are presented in Figure 3.2 and were unexceptionally positive. For the sock samples, the ATP test or protein test was not feasible due to the sampling technique. Visible soiling showed correlations with almost all other hygiene indicators used ($0.2 < r < 0.6$, $P < 0.01$). The *E. coli* load in the feeding equipment showed a correlation with only TCC ($r = 0.3$, $P < 0.001$). The ATP load from environmental samples was correlated only with the results from the rapid protein tests ($r = 0.3$, $P < 0.001$) and TVC ($r = 0.4$, $P < 0.001$). In contrast, the protein test and TVC results were correlated with TCC, *E. coli* and the absence of ESBL and ESBL *E. coli.*

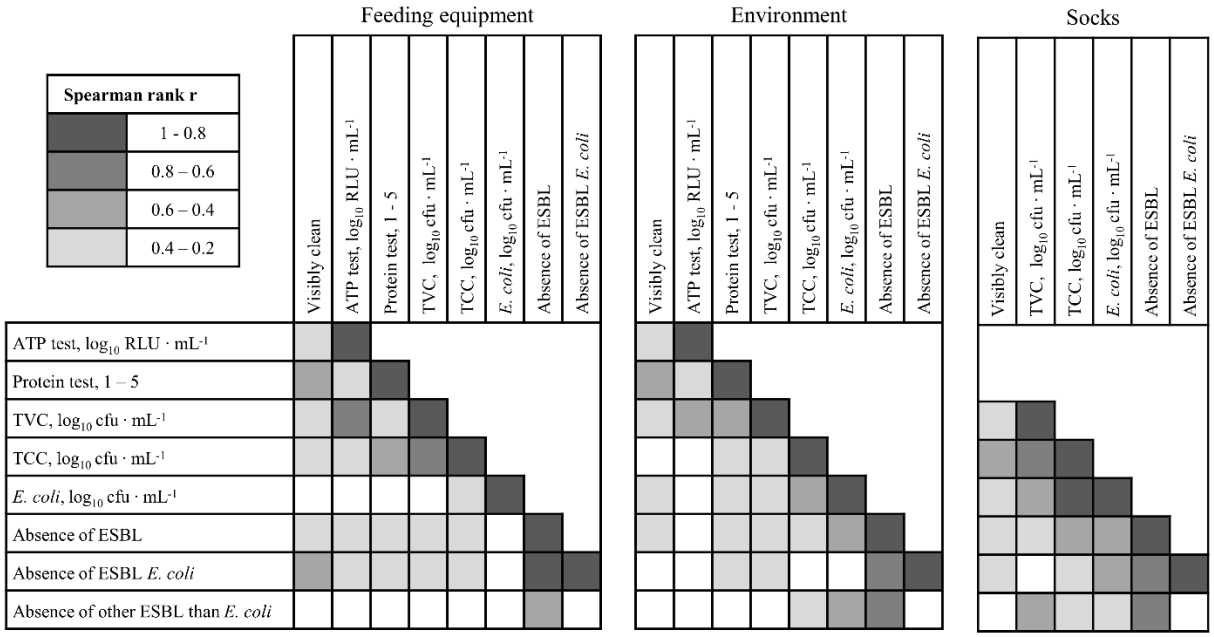

Figure 3.2. Spearman rank correlations ($P \leq 0.05$) between the hygiene indicators ATP, protein, aerobic total viable count (TVC), total coliform count (TCC), *E. coli*, extended-spectrum β-lactamase-producing bacteria (ESBL), ESBL *E. coli*, and other ESBL independent of the effects of sampling points.

3.5.3. Critical points in hygiene management

Only once did a bulk sample from the flooring test positive for *Cryptosporidium* spp. according to the enzymatic test but without microscopic confirmation of oocysts. Rotavirus and coronavirus were not detected after sanitation. All samples were negative for *Salmonella* spp. and MRSA. In 34 of 324 samples, ESBL were detected (10.5%), with 22 detections of ESBL *E. coli* (6.8%), 14 detections of ESBL *Acinetobacter* spp. or *Pseudomonas* spp. (4.3%) and 3 detections of ESBL *Klebsiella* spp. (0.9%). In four samples, more than one ESBL species was found after sanitation. ESBL species were predominantly found in sock samples (41.2%), followed by feeding equipment (35.3%) and environment samples (23.5%). At the farm level, eight out of the 11 farms were positive for ESBL, which also included all farms with reported diarrhea problems. The highest bacterial loads, expressed as the TVC, were found on feeding equipment and in sock samples (Fig. 3.3). Because of the different sampling techniques and on the basis of the scale findings, the sock samples could not be directly compared with other samples, so interpretation of the results must be considered with care.

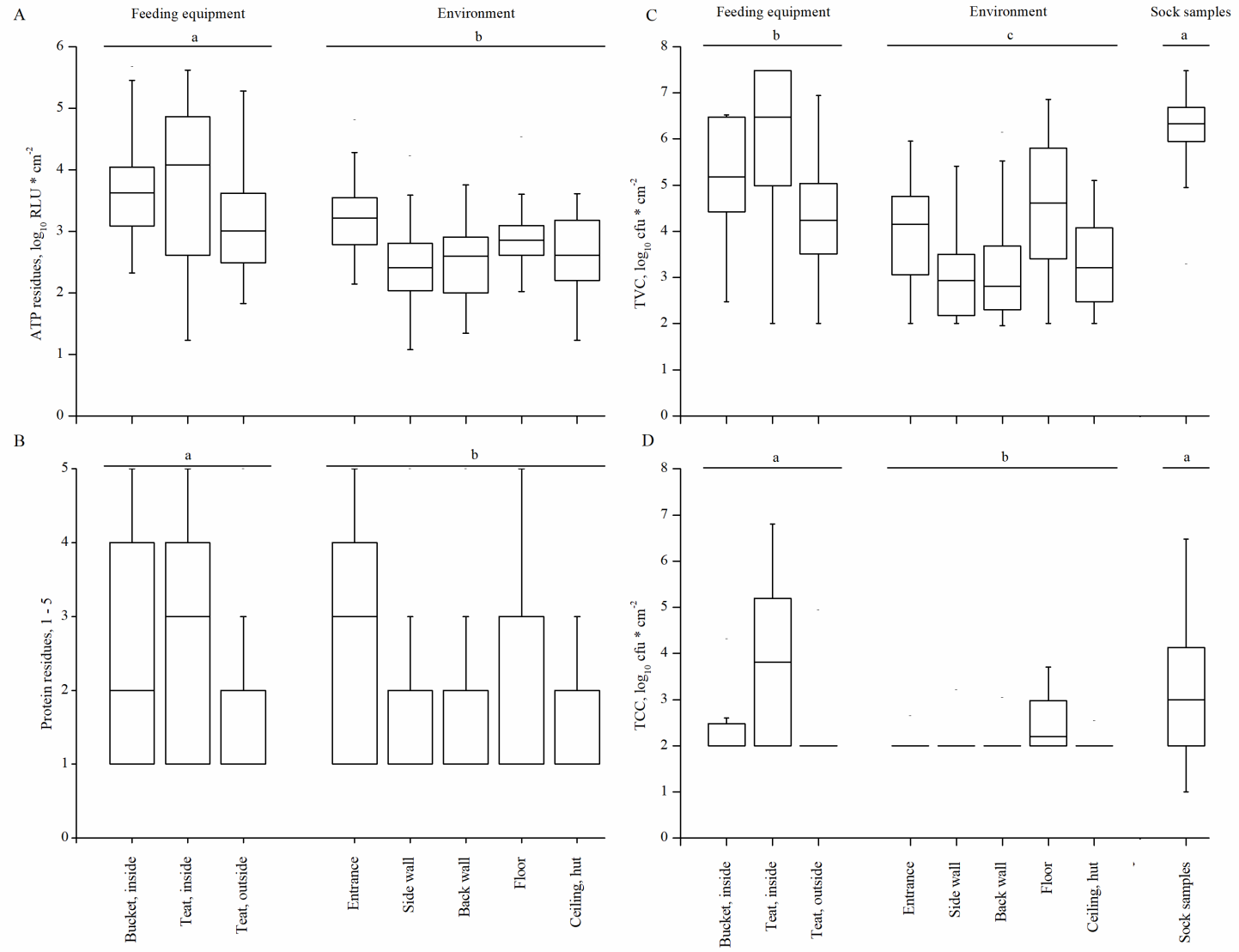

Figure 3.3. Results for ATP residues (A), protein residues (B), aerobic total viable count (TVC) (C), and total coliform count (TCC) (D) depending on the sampling areas: feeding equipment, environment or floor as done by sock samples. Different letters indicate significant differences ($P < 0.05$) between the sampling areas.

Environmental samples, primarily from the sidewalls and back walls, exhibited the lowest results for TVC, TCC, protein and ATP. TCC and ATP were highest on the feeding equipment, especially the inner surface of the artificial teat. The TCC was often below the detection limit of 2.0 $\log_{10}$ cfu · mL^{-1} in swab samples and 1.0 $\log_{10}$ cfu · mL^{-1} in sock samples (Table 3.2). Positive detections of TCC were obtained in 81.0% of the sock samples, 40.2% of the samples from feeding equipment and 11.5% of the environmental samples. The detection rate varied between the farms from 12.5 to 48.2% for TCC. In 80.5% of the sock samples, 5.1% of the samples from feeding equipment and 4.8% of the environmental samples, *E. coli* was detectable, with detection rates ranging from 3.1 to 24%.

Table 3.2. Positive results for coliform bacteria and *E. coli* and total number of samples (in brackets) from feeding equipment, the environment and sock samples from calf-rearing farms from the first (t 1) and second (t 2) samplings

Farm	No. of positive results for coliform bacteria						No. of positive results for *E. coli*					
	Feeding equipment		Environment		Sock samples		Feeding equipment		Environment		Sock samples	
	t 1	t 2	t 1	t 2	t 1	t 2	t 1	t 2	t 1	t 2	t 1	t 2
1	2 (6)	1 (6)	2 (8)	0 (6)	1 (2)	*	0 (6)	0 (6)	1 (8)	0 (6)	1 (2)	*
2	2 (6)	1 (6)	0 (7)	0 (8)	2 (2)	2 (2)	0 (6)	0 (6)	0 (7)	0 (8)	2 (2)	2 (2)
3	1 (6)	4 (6)	0 (8)	1 (4)	2 (2)	1 (1)	1 (6)	0 (6)	0 (8)	1 (4)	1 (2)	1 (1)
4	3 (5)	1 (2)	5 (8)	1 (8)	2 (2)	1 (2)	1 (5)	0 (2)	0 (8)	0 (8)	2 (2)	1 (2)
5	2 (6)	1 (6)	0 (8)	0 (8)	2 (2)	2 (2)	2 (6)	0 (6)	0 (8)	0 (8)	2 (2)	2 (2)
6	6 (6)	1 (6)	1 (7)	0 (7)	2 (2)	2 (2)	0 (6)	0 (6)	1 (7)	0 (7)	2 (2)	2 (2)
7	1 (6)	1 (6)	0 (8)	0 (8)	2 (2)	0 (2)	1 (6)	1 (6)	0 (8)	0 (8)	2 (2)	0 (2)
8	3 (3)	2 (6)	2 (6)	0 (6)	2 (2)	2 (2)	0 (3)	0 (6)	2 (6)	0 (6)	2 (2)	2 (2)
9	2 (3)	4 (6)	1 (9)	2 (10)	2 (2)	2 (2)	0 (3)	0 (6)	1 (9)	2 (10)	2 (2)	2 (2)
10	3 (6)	2 (6)	1 (8)	0 (8)	1 (2)	0 (2)	0 (6)	0 (6)	0 (8)	0 (8)	1 (2)	0 (2)
11	0 (2)	4 (6)	1 (8)	2 (8)	2 (2)	2 (2)	0 (2)	0 (6)	0 (8)	0 (8)	2 (2)	2 (2)

3.5.4. Risk factors for hygiene impairments

Risk factors that could have a negative impact on hygiene if they did not fit the expected demands for feeding equipment and housing equipment were estimated.
For feeding equipment, these tested factors were the feeding of waste milk, the feeding of high cell count milk, the lack of cleaning of feeding buckets after every use, the lack of disassembly of feeding buckets for cleaning, using only water for the cleaning of feeding buckets instead of cleaning with detergents and failing to use disinfectants when cleaning feeding buckets. For risk factors associated with housing, the evaluated variables were bright light, the absence of a slope to the back wall, the absence of cracks in the ground, smooth surfaces, the absence of contact between the calves, the use of individual pens or huts, rearing in huts, shifting of huts after use, daily changing of the bedding material, the use of detergents while cleaning, the use of disinfectants and regular disinfection of the pens after every calf. To determine the risk factors, odds ratios were calculated for the results of the different hygiene indicators used to

measure hygiene in feeding equipment samples (Table 3.3), environmental samples (Table 3.4) and sock samples (Table 3.5). Only significant risk factors (P-value ≤ 0.5) were considered. The use of detergents while cleaning feeding buckets resulted in higher visible cleanliness. Feeding of waste milk, feeding of high cell count milk and cleaning the buckets after every use were associated with a lower pass rate on the ATP tests (Table 3.3). Additionally, cleaning after every use decreased the odds for TVC, meaning a lower chance of being below the cutoff value. Disassembly of the feeding buckets, meaning unscrewing the artificial teat from the bucket prior to cleaning, resulted in higher odds of being below the cutoff values for ATP and TVC (Table 3.3).

Table 3.3. Results for risk factors with calculated odds ratios (ORs), 95% confidence intervals (CIs) and P-values for calf feeding equipment failing to meet the expectations.

Expectations	Percent failing to meet expectations, %	Total no.	OR	95% CI	P-value
Visibly clean					
Use of detergents	17.5	120	4.75	1.03 – 21.97	0.05
ATP test (3.5 $\log_{10}$ RLU · mL^{-1})					
Feeding of waste milk	17.1	119	0.41	0.18 – 0.92	0.03
Feeding of high cell count milk	47.1	119	0.29	0.12 – 0.70	0.01
Cleaning feeding buckets after every use	51.3	119	0.22	0.10 – 0.48	< 0.001
Disassembling feeding buckets for cleaning	47.1	119	2.20	1.01 – 4.83	0.05
TVC[1] (4.4 $\log_{10}$ cfu · mL^{-1})					
Cleaning feeding buckets after every use	67.8	118	0.34	0.15 – 0.79	0.01
Disassembling feeding buckets for cleaning	68.3	120	3.35	1.30 – 8.60	0.01

[1]aerobic total viable count

The management factor of the absence of a slope to the back wall indicates that fluids, such as cleaning water soiled with urine, feces or milk, could run off instead of contaminating the area where the calf lies, which is normally at the back wall. In environmental samples, the factor 'absence of slope to the back wall' increased the odds of visible cleanliness and the odds of being below the cutoff values for the protein test, *E. coli*, ESBL in all and ESBL *E. coli* (Table 3.4). The use of individual pens or huts, implying a greater distance between the calves, resulted in greater visible cleanliness and a greater likelihood of being below the cutoff value for the TVC. If huts were used on the farms, the factor 'shifting of huts' was recorded, with the hypothesis that shifting the hut to another place lowers the risk of soiling remaining from the last calf and the risk of vertical transfer of pathogens between calves. Shifting huts increased the odds of visible cleanliness and being below the cutoff value for the TVC. The absence of cracks in the ground leads to a lower amount of protein residues. The use of disinfectants,

independent of the frequency of usage, resulted in higher visible cleanliness and higher rates of being below the cutoff values for protein residues, the TVC and the TCC. Regular disinfection after every calf increased the odds of being below the cutoff value for protein residues. Smooth surfaces lowered the risk of detection of TCC and *E. coli* in environmental samples (Table 3.4).

Table 3.4. Results for risk factors with calculated odds ratios (ORs), 95% confidence intervals (CIs) and P-values for calf environmental samples failing to meet expectations.

Expectations	Percent failing to meet expectations, %	Total no.	OR	95% CI	P-value
Visibly clean					
Absence of slope to the back wall	19.2	167	7.28	2.12 – 25.00	0.01
Use of single pens or huts	19.2	167	3.93	1.12 – 13.77	0.05
Shifting of huts after use	13.4	112	12.12	1.50 – 98.01	0.02
Use of disinfectants	19.2	167	12.38	4.67 – 32.81	< 0.001
Protein test (3)					
Absence of slope to the back wall	29.3	167	5.56	1.58 – 19.63	0.01
Absence of cracks in the ground	29.3	167	2.11	1.02 – 4.35	0.04
Use of disinfectants	29.3	167	3.03	1.51 – 6.07	0.01
Regular disinfection of pens after every calf	29.3	167	2.48	1.12 – 5.17	0.02
TVC[1] (4.4 $\log_{10}$ cfu · mL^{-1})					
Use of individual pens or huts	25.9	166	4.36	1.44 – 13.17	0.01
Shifting of huts after use	25.9	166	2.34	1.05 – 5.20	0.04
Use of disinfectants	25.9	166	3.49	1.69 – 7.22	< 0.001
Total coliform count below detection limit					
Smooth surfaces	12.1	166	3.78	1.41 – 10.11	0.01
***E. coli* below detection limit**					
Absence of slope to the back wall	4.2	166	5.96	1.01 – 35.11	0.05
Smooth surfaces	3.6	165	10.75	1.96 – 58.83	0.01
Use of disinfectants	4.4	158	11.67	1.35 – 100.98	0.03
Absence of ESBL[2]					
Absence of slope to the back wall	4.8	166	9.93	2.02 – 48.87	0.01
Absence of ESBL[2] *E. coli*					
Absence of slope to the back wall	3	166	25.33	3.69 – 173.76	0.001

[1]aerobic total viable count

[2]extended-spectrum β-lactamase-producing bacteria

For 'rearing in huts' instead of 'rearing in pens', lower odds were seen for visible cleanliness, the absence of ESBL *Pseudomonas* spp. and *Acinetobacter* spp. in sock samples, as well as in regard to daily changes in bedding material and the TCC (Table 3.5). The absence of cracks in the ground increased the odds for being below the cutoff values for TCC and *E. coli* and for the absence of total ESBL, and the number of cracks in the ground affected the total ESBL detection in sock samples (Table 3.5).

Table 3.5. Results for risk factors with calculated odds ratios (ORs), 95% confidence intervals (CIs) and P-values for sock samples from calf individual housing pens failing to meet expectations.

Expectations	Percent failing to meet expectations, %	Total no.	OR	95% CI	P-value
Visibly clean					
Rearing in huts	46.3	41	0.09	0.02 – 0.45	0.01
Total coliform count below detection limit					
Absence of cracks in the ground	82.9	41	14.40	1.42 – 145.60	0.03
Daily change in bedding material	82.9	41	0.09	0.01 – 0.91	0.04
***E. coli* below detection limit**					
Absence of cracks in the ground	80.5	41	6.90	1.12 – 42.61	0.04
Absence of ESBL[1]					
Absence of cracks in the ground	34.2	41	6.46	1.15 – 36.45	0.04
Absence of ESBL[1] *Acinetobacter* spp. and ESBL *Pseudomonas* spp.					
Rearing in huts	17.1	41	0.06	0.01 – 0.61	0.02

[1]extended-spectrum β-lactamase-producing bacteria

3.5.5. Training effects

The results for the ATP, protein and TVC measures from the first and second visits were compared, depending on the farm, to observe a possible training effect (Fig. 3.4). In general, the training on individual farms showed limited time effects: only the levels of protein residues in feeding equipment and environment samples were significantly lower after training. Sock samples showed great variations in TVC and TCC between the first and second sampling without any consistent training effect but with time × farm interactions (Fig. 3.5). Almost all hygiene indicators differed among individual farms (Fig. 3.4 and 3.5). The proportions of samples above the detection limit for the TCC and *E. coli* in the feeding equipment, environment and sock samples were lower after training (Table 3.2).

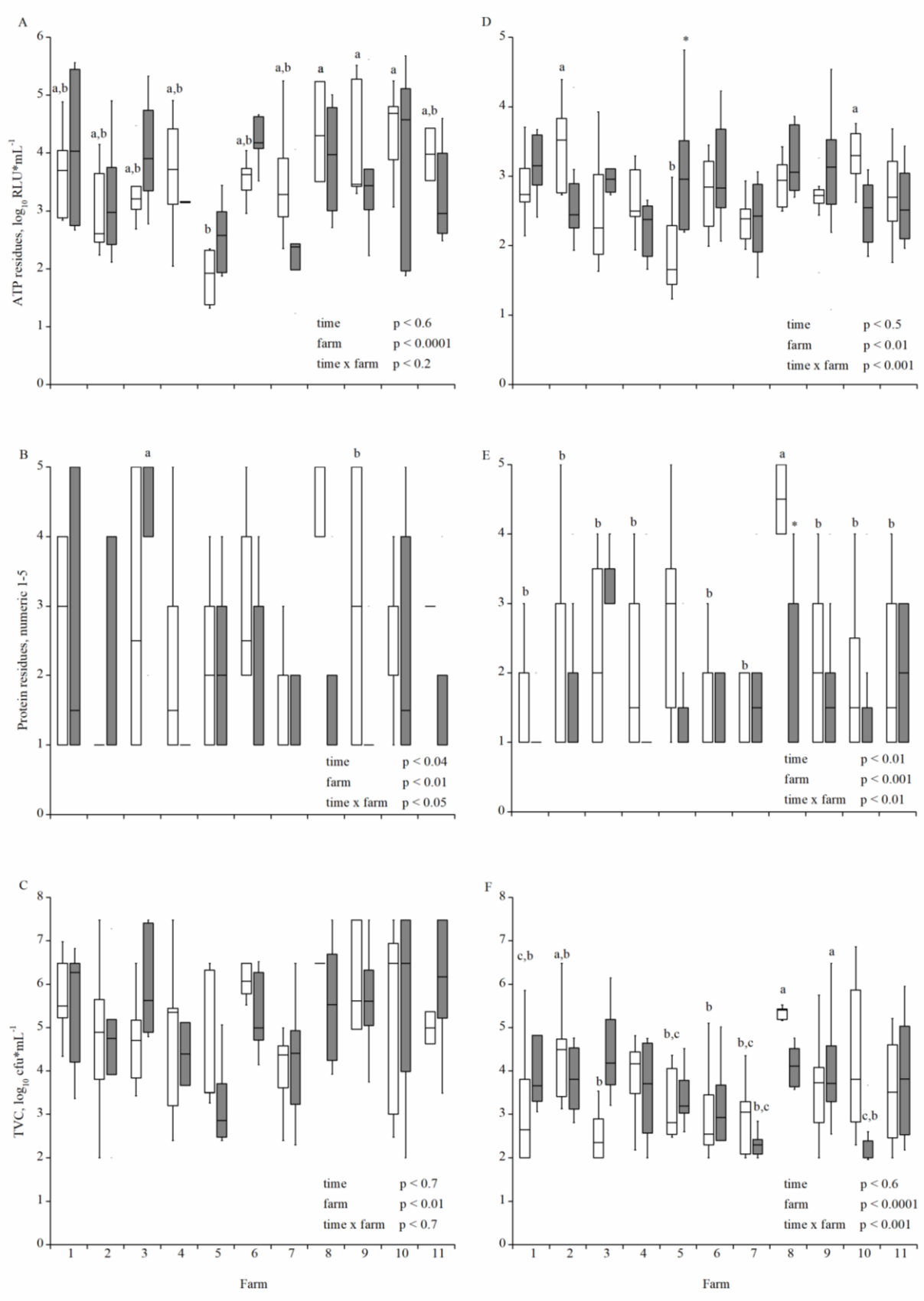

Figure 3.4. Results for ATP residues, protein residues and aerobic total viable count (TVC) from feeding equipment samples (A, B, and C) and environment samples (D, E, and F) from the first sampling (white boxes) and the second sampling (gray boxes) depending on the farm. Boxplots with different superscripts differ ($P < 0.05$).

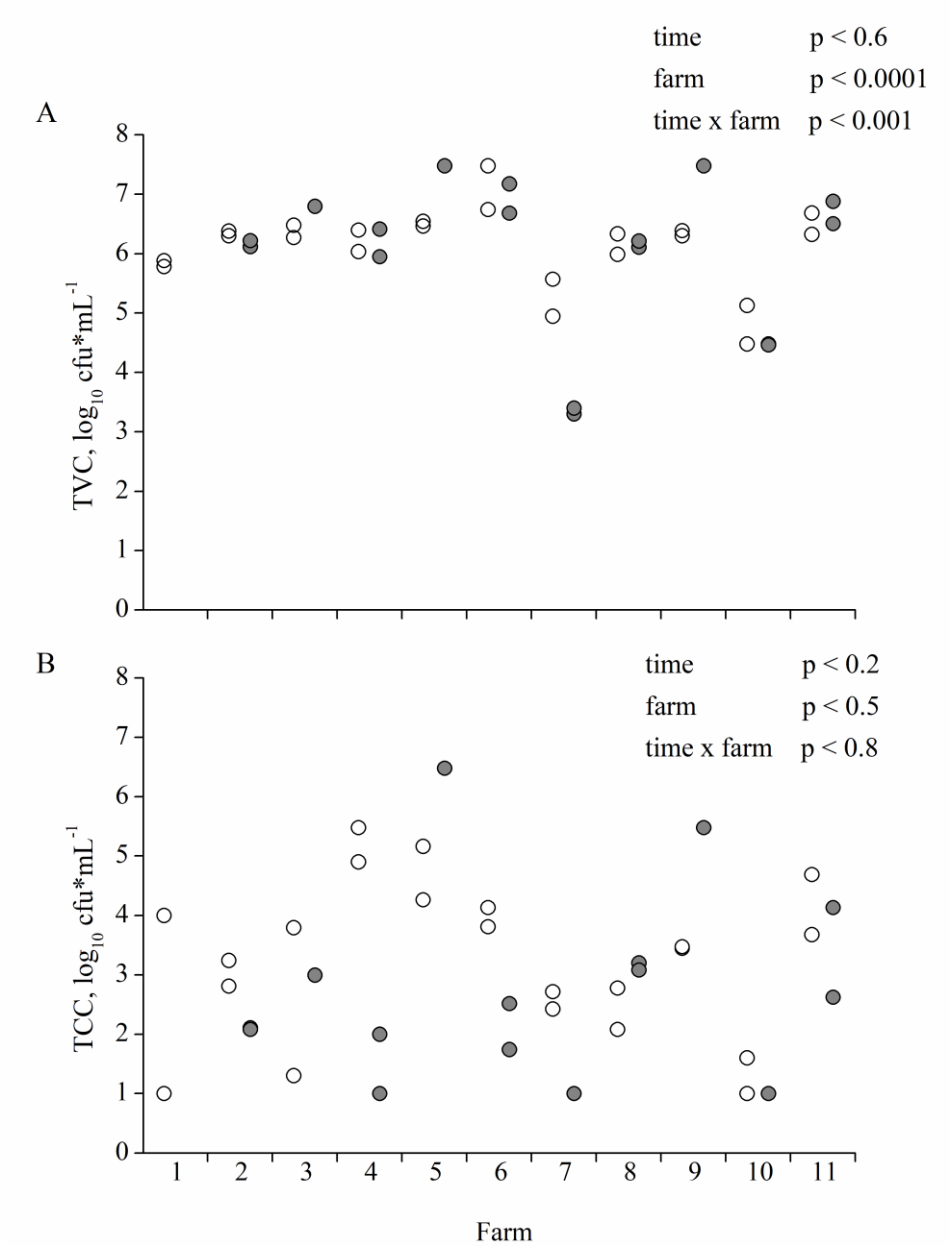

Figure 3.5. Results for aerobic total viable count (TVC) (A) and total coliform count (TCC) (B) from sock samples from the first sampling (white dots) and the second sampling (gray dots) depending on the farm. The results for the TVC and TCC of the second sampling on farm 1 were not analyzable.

3.6. Discussion

Healthy calves are the prerequisite for low antibiotic usage and economic success. For that purpose, hygiene plays a key role in maintaining calves' health. This study emphasizes a risk-oriented approach and the sampling of individual housing and feeding equipment after sanitation and preparation for restocking, which is a critical step in hygiene management. Maunsell and Donovan (2008) defined risk factors as those factors that reduced the ability of calves to resist diseases at a given level of pathogens and those that increased the level of pathogen exposure. In addition to appropriate colostrum management (Godden et al., 2009),

hygiene management is an important risk factor, as it prevents the carryover of diarrhea-causing pathogens. Reported calf losses within the first 14 days on the participating farms ranged from 0 to 5.9%, and only five farms reported a diarrhea rate > 5%, which seems low. Due to nonuniformly data recording in Germany differentiation between reasons of calf mortality such as stillbirth or diseases is not possible. The mortality rates vary between 10 and 15% in Germany and between 6 and 14% during rearing in other countries (Sanftleben, 2010; Johnson et al., 2011; Tautenhahn, 2017). Most likely, an underestimation occurred because the farms in our study had no valid data on calf mortality, so the rates were estimated. In addition, these farms participated voluntarily. Svensson et al. (2003) mentioned that farms participating voluntarily in scientific studies might be primarily well-managed farms. Even the reported incidence rate of calf diarrhea probably displays not the actual situation but reflects the self-awareness of the farmers of management problems. A relationship was observed between visible cleanliness, which is generally used by farmers to assess the level of cleanliness after sanitation, and the results for ATP, protein, TVC, the absence of ESBL and additional *E. coli* load in the environmental and sock samples. Even if bacterial or viral soiling is not necessarily visibly perceptible (Sherlock et al., 2009), it seems that a close visual inspection after cleaning and disinfection helps to identify weaknesses. This does not seem to be true for assessing the sanitation of feeding equipment based on visible cleanliness because it was not possible to draw conclusions about the presence of *E. coli*, ATP, or protein or the TVC. This shows the limits of relying on visible perception and might be the reason for the very high bacterial loads on feeding equipment.

3.6.1. Feeding equipment

The cleaning process for feeding buckets and artificial teats varied among the farms in regard to the cleaning frequency, temperature of the water and use of detergents. In a study from Austria, 97% of the investigated farms reported cleaning the feeding buckets after every use. Of these farms, only 25% used water with detergents while cleaning, whereas 25% cleaned with water alone (Klein-Jöbstl et al., 2014). This finding also corresponds to our results. The cleaning and disinfection of feeding buckets and teats is recommended after every use (Maunsell & Donovan, 2008), but according to our results, it is not implemented in practice in Germany. We found that 36.4% of the farms cleaned the feeding buckets after every use. In other studies, cleaning was reported more often, with 83.3% (Lundborg et al. 2005) or 77% (colostrum buckets) (Renaud et al. 2018). The feeding buckets showed the highest loads for all considered parameters (ATP, protein, TVC, TCC and *E. coli*). If there are diarrhea-causing pathogens in

the milk, it seems likely that they can survive and multiply in the buckets or in the artificial teat and will be ingested during the next feeding time. Furthermore, vertical transmissions can occur when feeding buckets are exchanged between the calves. Confusingly, the risk of ATP and TVC residues increases with the superficial cleaning of feeding buckets. This is probably because only rinsing with water without disassembling the teat only leads to an improved appearance, without improving the inner cleanliness. The ATP and TVC results were considerably better when the artificial teats were removed prior to cleaning. Unscrewing the teats from the feeding buckets during every cleaning is time consuming, which is often cited as a limiting factor (Gosling, 2018). Feeding systems with quick locks for artificial teats may help convince farmers to invest in better hygiene practices. Such systems are already commercially available but rarely used in practice, perhaps due to higher costs. The use of detergents to clean feeding equipment increased visible cleanliness and is already mentioned as a protective measure to reduce the prevalence of *C. parvum* (Trotz-Williams et al., 2008). Barry et al. (2019b) found no associations between feeding equipment hygiene and mortality rate. In their study, hygiene was assessed physically and by protein swabs. Both methods predominately reflect adhering dirt and feed residues and do not necessarily represent the bacterial burden. A considerable recontamination of pasteurized milk caused by irregular cleaning of milk taxis and feeding buckets in a study from Aust et al. (2013) led the authors to the conclusion that remaining pathogens from feeding equipment could counteract the positive effects of milk pasteurization prior to consumption. Bruning-Fann and Kaneene (1992) suspected a connection between calf mortality rates and the sanitation of feeding buckets. To avoid diarrhea or septicemia in newborn calves, proper hygiene of feeding equipment is crucial (Godden, 2008), and more attention should be paid to this topic.

3.6.2. Housing equipment

Individual housing is associated with lower risks of disease transmission and calf mortality (Svensson et al., 2003; Hotchkiss et al., 2015) and a lower burden of pathogenic factors (Barrington et al., 2002). The odds ratios for visible cleanliness and TVC below the cutoff value increased when huts were shifted between uses. The movement of calf pens is recommended by Hotchkiss et al. (2015) to reduce the enrichment of bacteria in the environment. The type of flooring seems to have an impact on *C. parvum* prevalence (Castro-Hermida et al., 2002, Trotz-Williams et al., 2008). We assume that this effect is transferable to other pathogens since concrete and other smooth surfaces are easier to clean and reduce the survival of pathogenic residues. This is in line with our results, showing lower ATP and protein residues and bacterial

loads and an increased odds ratio for the absence of *E. coli* on smooth surfaces. The absence of cracks in the ground leads to superior removal of TCC and *E. coli* and the absence of ESBL in sock samples. To avoid the accumulation of soiling and possible deposits of pathogens, farmers should take care of smooth surfaces and fix undesirable cracks. Direct exposure to sunlight is mentioned as a factor that decreases pathogens (Barrington et al., 2002), but we did not observe an association between bright sunlight and microbiological parameters. Daily cleaning of pens resulted in an 87% lower risk of infection with *C. parvum* in calves compared to a monthly cleaning interval (Castro-Hermida et al., 2002). Some tested farms in this study cleaned the pens after every calf, which is equivalent to an average interval of 14 days. Soaking with detergents resulted in significantly reduced counts of TVC and *Enterobacteriaceae* on metal and concrete surfaces and is recommended in livestock housing (Hancox et al., 2013). In our study, only a minority of farms used detergents to clean pens, and this was not associated with reductions in the levels of hygiene indicators. Bartels et al. (2010) found that consistent cleaning of calf housing areas was a protective factor against infections with coronavirus, which emphasizes the importance of proper sanitation. For the within-farm prevalence of *C. parvum* and cleaning of calf housing areas, no significant association was found (Trotz-Williams et al., 2008). Disinfection of the pens was part of the routine on the farms in this study. Odd ratios for visible cleanliness and meeting expectations on protein tests, for the TVC and for the TCC increased with the use of disinfectants after every calf. Disinfection led to significant reductions in the TVC and *Enterobacteriaceae* load on concrete surfaces in livestock housing (Hancox et al. 2013). In other studies, no associations between hygiene in calf pens and the occurrence of diarrhea were observed (Lundborg et al., 2005; Klein-Jöbstl et al., 2014).

3.6.3. Training effect and farm-specific practices

Farmers were informed about hygiene weaknesses after the first visit. In a personal conversation, it was found that most farmers were well aware of their weaknesses in cleaning and disinfection before the study. However, this awareness did not guarantee conceptual implementation and understanding of the consequences, as has been observed before (Lüdtke, 2004; Boersema, 2008). This might explain why information given in the training was only acted on at a few farms (based on the interaction) and translated into improvements in sanitation, contrary to what has been seen in pig fattening (Heinemann et al., 2020). Veterinary consultants should probably regularly draw farmers´ attention to the importance of hygiene in calf rearing and frequently point out weak points to achieve long-term changes. Differences among the farms also indicate that practical measures that are easy to implement are still

missing. Despite an extensive literature review, we could not find studies that are directly comparable to our study, as they have primarily focused on risk assessments in occupied pens, with hygiene as an additional factor. Since all-in all-out practice is well established in newborn dairy calf rearing, precise recommendations for proper sanitation or self-monitoring systems with practical hygiene plans, as is common for pig and poultry, are still rare. To the best of our knowledge, none of the reviewed studies dealt with combinations of visual cleanliness, rapid tests and microbiological measurements. Lundborg et al. (2005) mentioned that scientific data dealing with calf health and the effects of management and feeding procedures are surprisingly sparse, and Barry et al. (2019b) stated that hygiene practices in newborn calf rearing show substantial potential for improvement.

3.7. Conclusions

In conclusion, it should be noted that calf mortality can be caused by multiple factors, with calf diarrhea being the highest risk factor (Torsein et al., 2011; Johnson et al., 2017). Therefore, it is not possible to identify a specific factor since the various factors under field conditions affect each other (Bruning-Fann and Kaneene, 1992). To verify our results and the revealed risk factors, a greater number of farms should be visited, and fecal samples should be obtained from rehoused calves and analyzed for diarrhea-causing pathogens. Risk factors that impact individually housed newborn calves that were not included in this study but have been mentioned by other scientists, such as draughts, indoor vs. outdoor rearing, distance to walls, storage of milk buckets, individual milk feeding buckets for each calf, and slatted floors (Lundborg et al., 2005; Gulliksen et al., 2009; Klein et al., 2013), could be included in further investigations. In the long term, we can imagine that verified risk factors associated with hygiene could be part of a Hazard Analysis and Critical Control Points (HACCP) concept for calf rearing, as suggested by Boersema et al. (2008) and supported by supervising veterinarians in the prevention of calf diseases.

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4. Research Note: Tracing pathways of entry and persistence of facultative pathogenic and antibiotic-resistant bacteria in a commercial broiler farm with substantial health problems

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4.1. Abstract

On a commercial broiler farm with substantial health problems, shown by a reported loss rate of approx. 10 % and regular antibiotic use, samples were taken at different locations in two barns, with the aim of analyzing possible entry routes and persistence of pathogens and antibiotic-resistant bacteria as well as revealing weak points in sanitation. Therefore, swab samples for biofilm and water samples from animal drinking water lines and the spray cooling system were taken twice immediately before restocking. Additionally, swab samples from drain holes and air samples were collected. At restocking, hatchlings that died during transportation and chick paper were sampled. All samples were analyzed for the occurrence of *Pseudomonas aeruginosa*, total coliform count and antibiotic-resistant bacteria, namely, methicillin-resistant *Staphylococcus aureus* (MRSA), *Escherichia coli*, *Klebsiella* spp., *Citrobacter* spp., *Enterobacter* spp., *Acinetobacter baumannii*, *P. aeruginosa* and vancomycin-resistant enterococci (VRE). No MRSA or VRE were detectable. In all samples from drinking water and sprinkler system pipes, *P. aeruginosa* was detectable; in most cases, antibiotic-resistant *P. aeruginosa* was also detected, with varying resistance profiles. Samples from the hatchlings and chick paper were contaminated with antibiotic-resistant *Enterobacter* spp., with resistance to piperacillin, fosfomycin and the third-generation cephalosporines cefotaxime and ceftazidime. Therefore, the initial entry of antibiotic-resistant *Enterobacteriaceae* likely occurred via exposure at the hatchery resulting in colonization of the chicks. Animals on the fattening farm were treated with colistin, amoxicillin, and lincomycin in the last three production cycles prior to sampling. Due to the frequent administration of several antibiotic classes during the fattening period via piped water in both barns, resistance of isolates from water pipes accumulated, showing additional resistance to chloramphenicol and frequently to ciprofloxacin and levofloxacin. To prevent the development of secondary diseases caused by the facultative pathogen *P. aeruginosa* in chicks with weak immune status, the hygiene management for drinking water lines and the spray cooling system was changed. These changes resulted in an improvement in water line sanitation, shown by the absence of antibiotic-resistant bacteria and rare detection of *P. aeruginosa*.

Key words: poultry production, antibiotic resistance, water pipe, *Pseudomonas aeruginosa*, risk factor

4.2. Introduction

Frequent administration of antibiotics in livestock farming remains a problem, even if use as a growth promoter has been abandoned in the EU since 2006 and application in Germany has significantly decreased, provoked by the 16th Act to Amend the Medicinal Products Act (BMEL, 2019). In poultry, compared to that in other farm animal species, antibiotic usage remains high, reflected in a negligible decrease from 29.7 t in the second term of 2014 to 29.5 t in the second term of 2017 (BMEL, 2019). This lack of change is partly because health problems among broilers can rapidly lead to major losses due to the high total number of animals and high animal density, resulting in a high risk of infection. Therefore, hygiene in poultry production is essential for performance and animal health maintenance (Luyckx et al., 2015). Furthermore, a high standard of hygiene forms the basis for minimal antibiotic use (Gleeson and Collins, 2015). In the chicken fattening sector, cleaning and disinfection are frequently outsourced to cleaning contractors, with increasing tendency. Sanitation by cleaning contractors often leads to better results, probably caused by better knowledge and professional equipment (Maertens et al., 2018). However, farmers are still responsible for cleaning details, such as drinking water and sprinkler system pipes, which are sometimes neglected or insufficient, perhaps due to lack of time or deficient knowledge. The time allocated for sanitation is usually limited by an unchangeable scheduled delivery of new hatchlings, which explains why corrective measures are mostly impossible if unexpected challenges, such as delays in delivery of new hatchlings or transport of broilers to abattoirs, occur during sanitation. In addition to proper hygiene management, the chicks themselves constitute an important factor for later health and performance. The aim of this study was to determine the critical points of the entry and persistence of facultative pathogenic and antibiotic-resistant bacteria on a broiler fattening farm with substantial health problems after cleaning and disinfection.

4.3. Materials and methods

4.3.1. Farm characteristics and sample collection

Samples were taken at a commercial broiler farm located in North-Rhine Westphalia, Germany, with 79,000 fattening places for broilers distributed equally in two separate barns. Routine cleaning and disinfection of surfaces was carried out by a professional cleaning contractor. Cleaning was conducted with a high-pressure wash followed by disinfection with a disinfectant

consisting of glutaraldehyde and quaternary ammonium compounds. Cleaning and disinfection of the drinking water system was performed by the farmer with an alkaline cleaner containing sodium hydroxide and disinfected with a combination of peracetic acid, acetic acid and hydrogen peroxide. After disinfection, all the water of the drinking system was drained. During the fattening period, animal drinking water was disinfected continuously with a commercial product. The first sampling occurred 24 h before restocking, and the second sampling occurred immediately before restocking of the following production cycle to assess sanitation performance. Based on the results of the first sampling, hygiene measures were adapted as follows: reaction time for the detergent in the water drinking line was enhanced, with a subsequent thorough rinsing with fresh water. To avoid diluting the disinfectant effect, a drying time of 24 h was added prior to disinfection. Exposure time to disinfectants in the drinking water lines was enhanced from two to 12 h. The same procedure was implemented for the water sprinkler system. Additionally, the filters of the sprinkler system were disassembled and immersed in disinfectant solution for 12 h. Improvement of hygiene status was assessed in the second sampling, by comparing the results from the first and the second sampling. The farmer relay the results of previously conducted antimicrobial susceptibility tests, from both production cycles prior to sampling, from a contract laboratory. The farmer reported that all broilers were treated in the last three months prior to metaphylactic sampling with colistin, amoxicillin and lincomycin to reduce animal losses. In total, 26 samples of swabs, water, and air were taken in both barns. The following areas were sampled in each barn: six animal drinking water lines, two water sprinkler systems, one water sprinkler system filter unit, one dosing unit for medicinal products and nutritional supplements through drinking water lines, two drain holes, and one air sample (Fig. 4.1). Additionally, swab samples from drinking cups and feeding troughs from both barns were collected. For swab samples, sterile flocked swabs with 1 mL of liquid Amies medium (eSwab, Copa, Brescia, Italy) were used. Water samples were collected from areas of stagnating water in sterile tubes in 50 mL volumes. Collection of air samples was performed using a microbial air sampler (Coriolis micro, Bertin Technologies, Montigny Le Bretonneux, France) with 15 mL of sterile physiologic saline solution with 0.9 % sodium chloride (Oxoid, Basingstoke, UK) and a flow rate of 250 $L \cdot min^{-1}$ and 5 min sampling time, resulting in 1.25 m^3 of sampled air. The system aspirates the air and deflects it in the saline solution. Particles > 0.5 µm are deposited in the liquid and can be analyzed. Air samples were obtained in the center of the barns at a height of 50 cm above the ground. At the first restocking, nasal and cloacal swabs were collected from the three hatchlings that died during transport.

Three samples of chick paper from transport boxes were taken. Samples were transported in insulated boxes to the laboratory and analyzed within 24 h.

4.3.2. Microbiological analysis

The Amies medium from swab samples, water samples and air samples was directly analyzed without further dilution. A bulk sample of the three chick paper samples was created by weighing 5 g of each sample in blender bags with filter elements. In the filter bags, 145 mL of sterile physiologic saline solution was added. Samples were homogenized for 60 s with a bag mixer. All liquid samples were analyzed for total coliform count (TCC) (Chromocult coliform agar, Merck) as an indicator of fecal soiling via the pour plate technique. Plates were incubated at 37°C for 24 h. All blue and salmon-red colonies were counted as coliforms. For detection of *Pseudomonas aeruginosa*, cetrimide agar plates (Oxoid, Wesel, Germany) were used. Detection of antibiotic-resistant bacteria was performed with CHROMAgar plates (MAST Diagnostica, Reinfeld, Germany) for methicillin-resistant *Staphylococcus aureus* (MRSA) and for extended-spectrum beta-lactamase-producing bacteria (ESBL), namely, *Escherichia coli*, *Klebsiella* spp., *Citrobacter* spp., *Enterobacter* spp., *Acinetobacter baumannii*, *P. aeruginosa* and vancomycin-resistant *Enterococci* (VRE). The abbreviation "ESBL" was used for colonies that grew on ESBL plates and with resistance to third-generation cephalosporins. The plates were inoculated with 1 mL using the spread plate technique and incubated at 37°C for 24 h and 48 h according to manufacturer specifications. All cultural methods were conducted in duplicate. For testing susceptibility to antibiotics, suspicious colonies were subcultured on Columbia sheep blood (MAST Diagnostica) at 37°C for 24 h and identified by matrix-assisted laser desorption/ionization time of flight mass spectrometry (BioMérieux, Marcy-l´Etoile, France) with Myla software. Antibiotic susceptibility was tested via a microdilution assay using Micronaut-S MDR MRGN-Screening for gram-negative bacteria (MERLIN, Gesellschaft für mikrobiologische Diagnostika GmbH, Bornheim-Hersel, Germany). The results were interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical cut-off values for analyzing the resistance status of bacteria from the ESKAPE group (*Enterococcus* spp., *S. aureus, K. pneumoniae, A. baumannii, P. aeruginosa, Enterobacter* spp.) and *E. coli* of livestock origin against clinically important antimicrobials for humans.

4.4. Results and discussion

For systematic investigation, a representative number of samples of biofilms and water were taken from drinking water lines (swabs), drain holes (swabs), air (collected as a bulk sample), sprinkler system (water), filters of the water sprinkler system (water) and the dosing unit for medicinal products (water) and were qualitatively analyzed for the occurrence of ESKAPE bacteria and *E. coli* in the first sampling (Fig. 4.1). Both water samples from the dosing unit, as well as swab samples from the drinking cups and feeding troughs, were negative for all tested parameters. This result demonstrated that the water was fed into the drinking system without bacterial contamination and the high quality of the cleaning contractor. On broiler farms, feeding troughs and drinking cups seem to be of minor importance as critical points in sanitation, unlike those on pig fattening farms (Heinemann et al., 2020). Coliform bacteria were detectable in all water samples of the sprinkler system and filters of the sprinkler system. *P. aeruginosa* was found in all swab samples from the drinking water system from both barns (n = 12), both air samples, and all water samples from the sprinkler system and the filter unit (n = 4) but in only one of the swabs samples from the drain holes (n = 4) (Fig. 4.1). For water and air samples almost all samples exceeded the detection limit of 2.5 $\log_{10}$ cfu · ml^{-1}. Phenotypical ESBL *P. aeruginosa* were detectable in three swab samples of the drinking water lines and one sample of the sprinkler system in barn 1, in samples of the filter of the sprinkler system from both barns and in one swab sample of the drain hole in barn 2. The detection of phenotypical ESBL *Enterobacter* spp. (n = 2), *K. pneumoniae* (n = 2) and *A. baumannii* (n = 1) was less frequent. In all samples of the nasal swabs, in one cloacal swab of the hatchlings and in the bulk sample of the chick paper, ESBL *Enterobacter* spp. were detected. Additionally, ESBL *A. baumannii* was found in the chick paper sample (Fig. 4.1). In none of the analyzed samples were MRSA or VRE detectable.

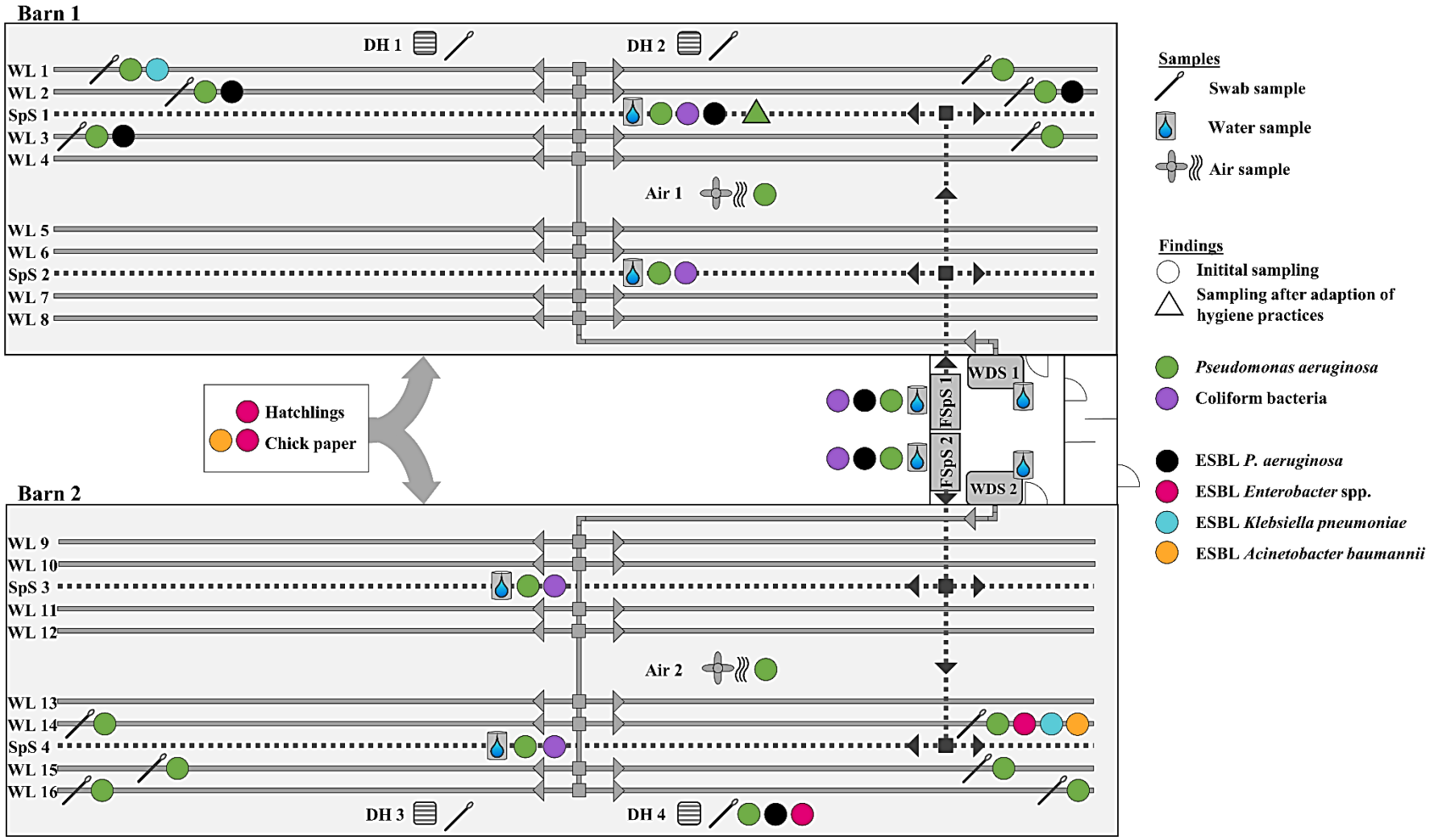

Figure 4.1. Swab samples were taken from animal drinking water lines (WL 1, 2, 3, 14, 15, and 16) from both sites and the pipes and drain holes (DH 1, 2, 3, and 4) in both barns on the chicken fattening farm. In the middle of both barns, air samples were collected (Air 1 and 2). Water samples were taken from the sprinkler system (SpS 1 and 2), the filters of the sprinkler system (FSS 1 and 2) and the water dosing units (WDS 1 and 2) of both barns. Additionally, cloacal and nasal swab samples from deceased hatchling and samples of the chick paper were analyzed.

These results indicate that the entry of resistant *Enterobacter* spp. and *A. baumannii* occurred via colonized animals from the hatchery, whereas on the sampled farm, *P. aeruginosa* originated predominantly from the drinking water and sprinkler system pipes. In previously performed antimicrobial susceptibility tests from a contract laboratory on three swab samples from yolk sacs immediately after hatching, *Enterococcus faecium* with resistance against trimethoprim with sulfadiazine, colistin, and tylosin and a reduced susceptibility toward enrofloxacin and penicillin was found. Also isolates of *Enterococcus faecalis* with resistance against trimethoprim/sulfadiazine, colistin, tylosin, and lincospectin, which consists of lincomycin and spectinomycin and a reduced susceptibility against penicillin were detected. In a sample from the pericardium of the hatchlings, *E. coli* was detected with resistance against amoxicillin, tylosin, and penicillin and a reduced susceptibility against lincospectin. The farmer reported that administration of antimicrobials, such as colistin and lincomycin resulted in insufficient recovery of the animals. The results of the antimicrobial susceptibility tests from the contract laboratory, explained why the antimicrobials that have been administered before the susceptibility testing, were inadequately effective. This circumstance emphasizes the importance of antimicrobial susceptibility testing prior to antibiotic treatment. In this study, neither *Enterococcus* spp. nor *E. coli* with antibiotic resistance were found in animal samples or in surrounding samples. The *Enterobacter* spp. isolates from hatchlings and chick paper all showed resistance to piperacillin and cefotaxime, ceftazidime and mostly to fosfomycin (5 out of 6 isolates) and intermediate reaction or resistance to ciprofloxacin (Fig. 4.2). The results for *A. baumannii* from hatchlings or chick paper were very similar, with additional intermediate resistance toward temocillin, the combination of piperacillin/tazobactam, and amikacin. One isolate of *A. baumannii* from a drinking water pipe showed resistance to almost all tested antibiotics except levofloxacin and a combination of trimethoprim and sulfamethoxazole (Fig. 4.2). Findings of extensively drug-resistant isolates are rare in farm animal samples and pose an alarming signal of extensive antibiotic usage. In the sprinkler system, the *P. aeruginosa* isolates showed resistance to cefotaxime, tigecycline, chloramphenicol and fosfomycin and a reduced susceptibility toward a combination of trimethoprim and sulfamethoxazole. One *P. aeruginosa* isolate was additionally resistant to ciprofloxacin and levofloxacin. *P. aeruginosa* isolates from drinking water lines showed increased diversity of antimicrobial resistance compared to that of the isolates from the sprinkler system (Fig. 4.2).

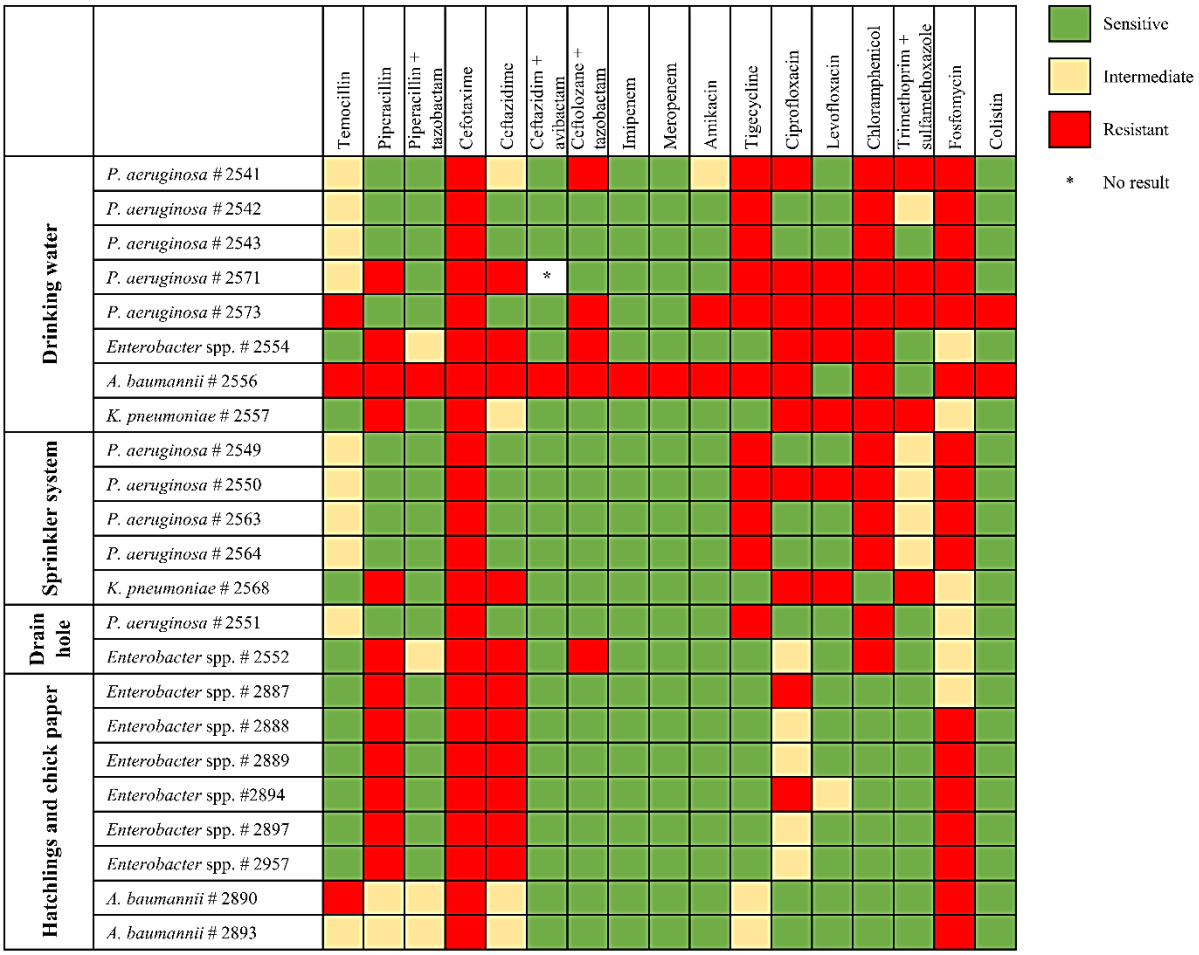

Figure 4.2. The results from antimicrobial susceptibility testing of the isolates from the chicken fattening farm showed variation depending on the organism and the origin of the isolate.

This finding is possibly caused by the administration of antimicrobials through the drinking water system, so antimicrobials acted as selectors for different resistances in the water lines. Additionally, due to the direct contact between drinking water lines and colonized animals, a direct exchange can occur, leading to adhesion of bacterial flora of the animals into the biofilm of drinking water pipes. This possibly explains differences in the resistance profile of bacteria from the sprinkler system and the drinking water lines. It seems obvious that the health problems of this farm were caused by two different factors. On the one hand, the chicks were already exposed to resistant bacteria, predominately *Enterobacter* spp., at the hatchery and arrived contaminated. On the other hand, *P. aeruginosa*-contaminated water was provided to the restocked chicks via the drinking lines and via mist by the sprinkler system from the first day in both barns. *P. aeruginosa* is known to form biofilms in aqueous environments, such as water pipes and siphons in animal and human environments (Sib et al., 2019). They usually persist long-term, causing recurring infections in chickens. Biofilm formation in water pipes is

a recurring process, especially after restocking when water flow is low, as pipes offer good growth conditions for bacteria. Application of vitamin supplements, which are often mixed with glucose, and other medicinal treatments via water lines delivers sufficient nutrients for bacteria and biofilm formation. The temperature in broiler barns also enhances bacterial growth in water pipes (Maes et al., 2019). Infections with *P. aeruginosa* occur mostly from environmental contamination (Wingender and Fleming, 2011). On this farm, the continuous spraying of mist contaminated with resistant and susceptible *P. aeruginosa* caused a major health problem. Usually, *P. aeruginosa* are opportunistic pathogens that lead to secondary infections when the immune status of chickens is already depressed and can cause septicemia, skin lesion infections, and hemorrhagic pneumonia (Gong et al., 2018). Young birds are more susceptible than older birds to infection with *P. aeruginosa*, but infections can occur at any age (Gerlach, 1994; Joh et al., 2005). As a consequence of the findings at the first sampling, more specific hygiene measures, like enhancing the exposure time for detergents and disinfectants in water lines or disassembling of the water filters were implemented on the farm, as described above. The effect of the adopted measures could be seen by the results of the second sampling, where the analyzed ESBL and coliform bacteria were all below the detection limit of 1.0 $\log_{10}$ cfu · ml^{-1}. Only in one sample from the sprinkler system was *P. aeruginosa* persistent. The results from this case study emphasize the importance of proper hygiene management to reduce antibiotic usage and the spread as well as the development of antibiotic resistance. The antibiotic resistance pattern that had already been acquired in the hatchery remains a major problem in fattening farms and needs to be addressed in future investigations (Projahn et al., 2016). Therefore, the aim should be to minimize the conscious use of antibiotics in broiler breeder farms to avoid early entry of resistance to the broiler meat production chain. Regarding extensively drug-resistant *A. baumannii*, further investigations on this farm should be done to prove whether this animal and human health-threatening strain was stably eliminated by sanitation improvements. In conclusion, systematic investigations by sampling not only the chicks but also the barns before restocking helps to uncover critical points in hygiene and might be used as a basis for consultation or as a service of the cleaning contractors to successfully eliminate potential pathogens by implementing targeted measures.

4.5. Acknowledgements

Declarations of interest: none.

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This study was conducted in accordance with federal and institutional animal use guidelines (GER, NRW, Az. 84-02.05.40.16038), a data privacy agreement (University of Bonn, 38/2018) and ethical standards.

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5. General discussion and conclusion

It is key to ensure proper cleaning and disinfection practices in order to interrupt infection chains, prevent the spread of pathogenic and antibiotic-resistant bacteria and to maintain animal health, animal welfare, quality of food animal origin and thereby also protect consumer health. Good management is an important factor in implementing a high standard of cleaning and disinfection, which is a major part of hygiene in farm animal housing. To establish a good hygiene management, it requires suitable and reliable indicators to evaluate the performance and efficiency of cleaning and disinfection. Further, knowledge of critical points in sanitation is needed in order to improve processes systematically. Ultimately, improvements can only be achieved if farmers have, on the one hand, knowledge of the critical points and methods for checking the hygiene measures and are, on the other hand, willing to adapt their management accordingly. Therefore, it is important to implement scientific findings in practical recommendations.

In the course of the here presented studies, different hygiene indicators were tested in pig fattening stables (chapter 2) and dairy calf rearing facilities (chapter 3). One of the aims of both studies was to identify appropriate methods for monitoring the success of hygiene measures in animal production and to detect potential influencing variables.

Visual evaluation is usually the only method for assessment of proper cleaning in animal production. It is a simple and cost-effective measure, but is often criticized as unreliable and inaccurate in studies from the healthcare sector (Lewis et al., 2008; Sherlock et al., 2009; Mulvey et al., 2011), the food industry (Cunningham et al., 2011) as well as animal production (Huneau-Salaün et al., 2010; Luyckx et al., 2015). It is obvious that the visual impression cannot reflect the content of bacteria or even the presence of pathogens on a surface. Nevertheless, a critical visual assessment is an important first indication of the cleaning success. If debris is still visually perceptible, it is rather unnecessary to disinfect the surface since the disinfectant will mainly interact with organic residues. To enhance the visual evaluation of remaining debris, surfaces can be wiped with a white cloth or a white cotton swab for drinkers or pipes. Gosling (2018) recommended the use of white moist wipes and a powerful torch. An even more specific visual evaluation is possible with a fluorescent chemical tracer, which is used in hospital settings. However, it is rather unsuitable for the use in livestock farming, as completely uncleaned areas are usually clearly perceptible, and the usage of a high-pressure washer will remove these water-soluble marks immediately.

The use of the protein rapid test, which in principle is also just an instrument for visualizing contamination at critical points, showed positive correlations to visual assessment in samples from pig fattening farms (chapter 2) and calf rearing facilities (chapter 3). It is a conceivable method for training purposes and has the advantage of reflecting more specific results compared to visual evaluation, as the results are independent of lightning conditions, the color of assessed materials and subjective perception. However, for long-term monitoring and the implementation of a quality control chart protein rapid tests are less suitable due to their limited and therefore imprecise scale. The noticeable color change in used protein rapid tests, visualizes cleaning results and plays a major role with regard to the learning effect. Visualizing issues helps to remember certain aspects in the long term. This was also shown in studies from the healthcare sector (Goodman et al., 2008; Mitchell et al., 2015).

Results of ATP rapid tests indicate both dirt residues and bacterial contamination, in contrast to protein tests or visual evaluation, which only indicates soiling and can therefore to a certain extent also provide information about the success of disinfection measures. The usefulness of ATP rapid tests for hygiene control has been evaluated very critically in some studies. This is largely due to the fact that the results of ATP tests in the reviewed studies are often directly compared to the results for TVC, which is the standard procedure for testing the hygiene status of surfaces. For example, Sherlock et al. (2009) could not find a significant correlation between the measured ATP values and the TVC from swab samples. Whiteley et al. (2015) conclude that ATP rapid tests show very strong scattering and only low precision, whereby their study does not specify which bacterial concentrations were tested. Many other authors were able to show a positive correlation between ATP results from rapid tests and the measured TVC (Cunningham et al., 2011; Osimani et al., 2014; Clemensson et al., 2018; Öz & Arun, 2019), which was confirmed for samples from pig fattening farms and calf rearing facilities in the presented studies. When considering whether ATP tests are suitable for use as hygiene indicators on farm, it should be recognized that the systems are only rapid tests. Even if the displayed ATP content does not necessarily correlate with the TVC and even less with the content of certain pathogens, the information is still available on time and relevant for management decisions, because a high ATP content generally indicates the presence of residues that can form a nutrient medium for microorganisms. In order to conclude whether ATP rapid tests are suitable for use in farm animal housing, it should always be considered that the hygiene requirements in the healthcare and the food sector are substantially higher. For example, in studies from the healthcare sector 100-500 RLU are given as cut-off values (Lewis et al., 2008;

Sherlock et al., 2009; Mulvey et al., 2011), although it is not stated whether the RLU values refer to the size of the sampled area or are to be interpreted per cm^2. Casini et al. (2018) recommend cut-off values of 50 RLU · 100 cm^{-2}. Osimani et al. (2014) suggest values from 100 to 400 RLU · 100 cm^{-2}, depending on the cleanability of the sampled area. The results for ATP values from fattening pig and calf housing varied between 1 and 9,340 RLU · 100 cm^{-2}, depending on the sampled area. This major difference in the results from the different studies underlines the importance of defining separate cut-off values for each area. In some studies, it is mentioned that ATP rapid tests are very simple, indicating that no extensive basic knowledge is required for the application (Andersen et al., 2009; Casini et al. 2018). This cannot be confirmed as ATP rapid tests are quite susceptible to interference and can interact with disinfectant residues, which leads to quenching or enhancing of the measured emitted light, depending on the respective chemical agent. The test should therefore only be used on dry surfaces (Alfa et al., 2015), which also applies to protein rapid tests. In routine use, unusual values or strong deviations from the common value should therefore be critically checked. Although ATP rapid tests are a fast and effective tool for determining the hygiene status and the success of sanitation measures as shown in samples from pig fattening farms and calf rearing facilities, the initial cost of the luminometer, which is needed to evaluate the tests and the cost of each test, makes this hygiene indicator rather unsuitable for farmers themselves. Nevertheless, the investment in a luminometer and the use of ATP rapid tests could be a useful additional service by consultants or veterinarians during advisory visits at farms to improve their hygiene management. It is also conceivable as an audit tool or for the self-assessment of cleaning companies entrusted with the cleaning of animal housing facilities.

In order to make specific statements about the success of disinfection and thus the reduction of microorganism, microbiological tests are necessary. In many studies, ACP are used for the microbiological examination of surfaces. These have the advantages that they are easy to process, fast to apply and time-efficient (Lutz et al., 2013; Luyckx et al., 2015). The application of ACP is standard in the healthcare sector and food industry, even if the recovery rate is controversially discussed in several studies (Cunningham et al., 2011; Lutz et al., 2013). As shown in the previous chapters 2 and 3, ACP have been proven to be inapplicable for many sampling areas in livestock housing. Sampling of concrete floors, which often have a rather rough structure leads to the destruction of the soft agar surface. In animal housing, dirt particles and dust are often still present after cleaning, which adhere to the agar surface during sampling and thus hinder the growth of bacteria and also make interpretation of the results more difficult.

This was also found by Huneau-Salaün et al. (2010) for investigations in layer houses and Lutz et al. (2013) in a laboratory simulation. The determination of the TVC using ACP with a non-selective medium also turned out to be not feasible in pig housing, as most of the ACP were overgrown and could not be evaluated. In livestock housing often fungal spores or swarming bacteria, such as *Proteus* spp. are still present, which overgrow the entire agar surface and thus make evaluation impossible. A statement whether ACP are time-consuming and expensive (Cunningham et al., 2011) or time-efficient and require only low cost, due to the low effort for further processing (Lutz et al., 2013), depends on the methods with which they are compared. In conclusion, the use in farm animal housing is rather limited. Therefore, the recommendation of ACP for the control of cleaning performance in the DLG guidelines (Von der Von der Lage et al., 2010) should be regarded rather critically. The application of ACP for hygiene control of bulk milk tanks and similar smooth equipment, made of stainless steel, with correspondingly high hygiene requirement might be conceivable.

The results of microbiological swab methods for determining the TVC or for the detection of pathogenic bacteria are generally better evaluated (Lutz et al., 2013; Casini et al., 2018). For pig fattening farms and calf rearing facilities positive correlations of the TVC with other microbiological parameters, as well as results from ATP rapid tests and visible evaluation and protein tests were found. When interpreting the results for TVC, it should be noted, that a high total bacterial count can only provide limited information about a specific risk of infection (Sherlock et al., 2009; Mulvey et al., 2011). For application in the stable, swabs are to be preferred to the ACP method, as these can also be used in difficult areas and are independent of the roughness of the floor or adhering dirt particles. In addition, several bacterial species can be detected from the same sample by analyzing the sample liquid.

For sampling of larger areas, sock samples are an alternative method. However, due to the different sampling approach, it is not possible to compare the results of the sock samples directly to the results of, for example, ATP tests. Since the number of steps can be adjusted to cover a large area, they are suitable as indicators of general cleanliness and especially for the detection of rather uncommon pathogens, like antibiotic resistant bacteria. Lutz et al. (2013) used antistatic cloths for the sampling of larger areas, which might be an interesting option for further investigations.

The choice of a suitable test system depends primarily on the problem posed. For determining the success of cleaning, rapid tests and, above all, an initial evaluation by visual inspection are appropriate options. However, when the aim is to check the reduction of microbial load, i.e. the

success of disinfection procedures, or the elimination of a specific pathogen, microbiological testing using swabs is still the gold standard as shown in chapters 2 and 3. Mitchell et al. (2013) made similar conclusions for the evaluation of cleanliness in the healthcare sector.

In order to monitor the success of implemented hygiene measures, it is not only necessary to select the appropriate hygiene indicator but also to be aware of the particularly critical points in routine cleaning and disinfection of farm animal housing. Identified critical points in fattening pig housing are primarily the drinkers. There, high TVC were still detected after cleaning and disinfection. In addition, the troughs were often insufficiently cleaned, as presented in chapter 3. Mannion et al. (2007), Gonzalez et al. (2015) and Luyckx et al. (2016) also found that drinkers and troughs in pig farming were still highly contaminated after cleaning and disinfection and had an impact on the spreading of Salmonella. During the farm visits for the presented study in chapter 2, it was also noticed that some of the feeding troughs for pigs still contained rinsing water and disinfectant residues, which can lead to an impairment of the pigs' health. During the evaluation of the critical areas in pig stables, it was also noticed that the outside of pipes for feed supply or the lower edges of troughs were often not cleaned, probably because they are outside the direct field of view.

In calf housing, a high total microbial count was still detectable, especially in the milk feeding buckets and artificial teats, and frequently also a contamination with coliform bacteria, including *E. coli* and occasionally ESBL. The detection of *E. coli* and ESBL might be caused by feeding waste milk from cows suffering from mastitis or treated with antibiotics. This practice could lead to an increased shedding of antibiotic resistant bacteria from the calves, as shown by Aust et al. (2013). While cleaning, care should be taken that milk feeding buckets for calves are cleaned with separate brushes that are only used for this purpose in order to prevent the transfer of pathogens from older animals.

On poultry farms, a high awareness of the importance of cleaning and disinfection measures is already ensured by the legal requirements. This awareness was also observable in the case study presented in chapter 4. This study was planned after a request by a farmer whose chickens of several runs had continuously been suffering from a massive health problem and were therefore prophylactically treated with antibiotics shortly after housing. In order to solve this health problem, microbiological swab, water and air samples were taken throughout the stable after cleaning and disinfection. High levels of TVC and especially facultative pathogenic *P. aeruginosa* were found in the drinking water lines and in the sprinkler system pipes. *P. aeruginosa* belong to the typical biofilm formers in aqueous systems and are often found in

water pipes (Groß, 2013; Sib et al., 2019). Additionally, several species of ESBL were detected. Critical values for bacterial load in drinkers in poultry houses were also found by Luyckx et al. (2015). At the visited chicken fattening farm, a professional company was hired to clean and disinfect the barn, which generally led to a high hygiene standard, shown by a low TVC and the absence of *Pseudomonas* spp., *E. coli* and other coliform bacteria for drinking cups and floor samples. However, the cleaning of the drinking water pipes and the sprinkler system was the farmer's responsibility and was conducted rather carelessly. It is most likely that the increasing contamination with *P. aeruginosa* in the drinking water pipes and the sprinkler system led to the so-called phenomenon of barn fatigue. According to Sommer et al. (1991), together with the increasing pathogen pressure of facultative pathogenic bacteria, the virulence of them also increases with an increasing number of host passages and leads to a rising rate of diseases. Additionally, to the poor hygiene of the water pipes from the drinking water and the sprinkler system, the housed chicks came from the hatchery already colonized with antibiotic-resistant bacteria. Subsequent to the farmer's complaint to the hatchery, the chicks of the next runs were significantly more vital, which suggests that the hatchery is well aware of the problem. The health status of the subsequent run was improved by a targeted adjustment of the cleaning and disinfection measures with a focus on the water carrying systems prior to rehousing. According to the farmer, it was possible to avoid the treatment with antibiotics completely. Although only one farm was considered for the case study, the results support the general argument that inadequate cleaning can lead to an increased risk of disease outbreaks. Additionally, the results confirm the hypothesis that optimized hygiene can reduce the incidence of diseases.

In all the investigations carried out independently of the animal species, it was noticeable that the drinking and feeding facilities often showed high bacterial loads after cleaning and disinfection. As the animals have direct contact to these areas with the mucous membranes during water and feed intake and might ingest potential pathogens directly, this should be viewed critically. To conclude, more attention should urgently be paid to improve cleaning and disinfection of the feeding equipment and drinking facilities, resulting in an entirely improved hygiene status.

In addition, to the evaluation of hygiene indicators and the identification of critical points in sanitation, it is essential that scientific findings are transferred into practice and that recommendations are also implemented by farmers. In general, it could be stated that the problem of cleaning and disinfection is less a lack of knowledge, but rather a lack of

communication, available time and effort. Farmers are aware that regular cleaning and disinfection is required by law, or at least strongly recommended in the case of dairy farming. Nevertheless, the success of hygiene measures depends highly on the training and motivation of farmers and employees as well as on the right approach of cleaning (Smulders, 2007). To improve the training of the farmers, the results from the first samplings and the evaluated critical points on pig fattening farms and calf rearing facilities were presented and discussed with the farmers. The sampling was repeated to evaluate a possible improvement, due to a training effect. A training effect, shown by lower rates for almost all tested hygiene indicators, was measurable for pig fattening farms. For calf rearing on dairy farms no consistent training effect was observable. The results showed great variations depending on the farm. This indicates that pig farmers are more aware of the importance of proper hygiene. Especially in dairy farming, the importance of cleaning and disinfection has apparently not been realized yet, which was partly implied by the reactions of farmers in discussions during sampling of calf housing and feeding equipment and is perhaps due to the lack of legal specifications. Kühl (2007) also comes to this conclusion for dairy farms in general. However, a positive effect of sanitation measures for dairy cows has already been shown: For example, a regular cleaning of the barn alley floor improved the cow hygiene shown by positive correlations between visual cleanliness of the barn, udder hygiene as well as condition and cell count in milk (DeVries et al., 2012). Perhaps a cost-benefit analysis would be useful in communicating the importance of the issue, providing farmers with concrete data on what impact the improvement of hygiene measures and maintenance of performance standards can have. The advice of the farm veterinarian is also a key factor in improving hygiene management on farms. Pointing out critical points regularly could perhaps remedy the situation. In the discussions with the farmers it was also found that the disinfectant list of the German Veterinary Medical Society (Deutsche Veterinärmedizinische Gesellschaft, DVG), which lists tested commercial disinfectants, the recommended concentration for application and exposure time and, above all their, spectrum of action (bactericidal, fungicidal, virucidal, antiparasitic), was partly unknown to the farmers. It was also partly unfamiliar that the agents used were not equally effective against all microorganisms or that the agent should be changed occasionally to avoid the development of resistances. Apparently, there is still a need for clarification in this respect. In the case of the particularly contaminated drinking water pipes in the broiler house, the instructions for use of the applied disinfectant stated that the agent showed a good dissolving effect against biofilms at an exposure time of 60 minutes. As this did not result in the desired success, the manufacturer

advised to increase the exposure time to more than 12 hours. Such statements are somewhat surprising and can lead to uncertainty concerning the correct procedure.

As a result of the farm visits during the different studies, specific approaches were found to solve some of the problems related to the farm management of the considered animal species. For practical implementation, in order to avoid rinsing water in feeding troughs of pig farming, it would be useful if the troughs had an outlet and could be emptied completely, which was not the case on all farms. When asked, one farmer stated that the residues were always removed manually with a cloth, which is both time-consuming and poses a considerable risk of contamination via the used cloth. The hand hygiene of the staff should also be viewed critically, especially in the area of piglet rearing. Here, regular hand hygiene or possibly a regular change of disposable gloves, e.g. during castration, would be useful in order not to spread possible pathogens between piglets from different pens (Fotheringham, 1995).

In calf housing, regular cleaning of the milk feeding buckets should be improved. Especially the fact that not all farmers unscrew the artificial teats from the feeding buckets regularly for cleaning must be critically evaluated. Currently, dairy farmers are often overworked and stressed, resulting in a lack of available time and motivation to invest in additional work (Hansen & Østerås, 2019). But, in order to save time, it is key to ensure each step is carried out effectively and efficiently the first time to reduce the need for repeating the process. (Gosling, 2018). The health and welfare of dairy cows is paramount, especially since the present profit margin for calves is extremely low, which might be another explanation for a rather low motivation to clean feeding buckets. To save time, it can be helpful to replace them with milk feeding buckets with a quick release of the artificial teats. Nonetheless, the artificial teats should be changed regularly. Individual buckets for each calf could help to prevent the horizontal spreading of diseases among calves (Fotheringham, 1995). This would be easiest to achieve if the pens or huts for individual calf housing and the buckets were numbered consecutively to ensure a clear assignment to each calf. In addition, care should be taken to ensure that the buckets are not stored in the milking parlor, as contamination is often caused by the splashing feces of the cows during milking. The fact that the highest levels of bacteria were always found in feeding and drinking equipment is probably related to the aqueous environment, in addition to the input of bacteria by the animals and the provision of nutrients for the bacteria. Desiccation can have a greater effect on the surviving of bacteria than disinfection (Asséré et al., 2008; Hancox et al., 2013). Therefore, feeding buckets should be stored upside down in a dry environment after cleaning to improve drying.

To improve the hygiene of water pipes, the use of an endoscope camera is appropriate to determine biofilms and performance of sanitation measures. However, it should always be remembered to disinfect the camera thoroughly afterwards to avoid carry-over of bacteria. In cases of high contamination of water pipes, the reaction time of detergents and disinfectants should be extended, in consultation with the producer. Another idea to reduce biofilm growth and to improve the water quality might be the additional equipment of the water pipes of drinking lines and sprinkler systems with antimicrobial effective coatings, as this is assumed to be effective in the food sector (Dohlen, 2016).

In conclusion, successful cleaning and disinfection as part of farm hygiene management can only be achieved with reliable indicators to identify critical points in sanitation and the knowledge and willingness of farmers to implement new measures. In the course of this thesis it was possible to show which hygiene indicators are suitable for use in animal production. Feeding equipment and drinking facilities were identified as a reemerging weak point. However, in order to successfully transfer this knowledge to farmers and establish an efficient hygiene management, inter-farm cooperation with veterinarians and advisors as well as recurring educational activities are required.

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6. Annex

6.1. Supplemental information to Chapter 2

Supplemental table 6.1. Surface materials and roughness of the sampled areas on the pig fattening farms. The availability of sampled areas determines the number of total samples.

	Farm 1	Farm 2	Farm 3	Farm 4	Farm 5	Farm 6
Sample point	Material					
Entrance door, inside	Plastic[0]	Wood[1]	Plastic[0]	Plastic[0]	Plastic[0]	Plastic[0]
Back wall	Plastic[0]	Concrete[1]	Stainless steel[0]	Tile[1]	Tile[0]	Concrete[1]
Side wall	Concrete[1]	Concrete[1]	Plastic[0]	Plastic[0]	Plastic[0]	Plastic[0]
Ceiling	Plastic[0]	Wood[1]	*	Plastic[0]	Plastic[0]	Straw plates[1]
Slatted floor	Concrete[1]	Concrete[1]	Concrete[1]	Concrete[1]	Concrete[1]	Concrete[1]
Manure area	Plastic + Concrete[1]	Wood[1]	Stainless steel[0]	Tile[1]	Plastic[0]	Stainless steel[0]
Feeding area	Concrete[1]	Rubber[1]	Rubber[1]	Concrete[1]	Concrete[1]	Rubber[1]
Feeding tube, upside	Plastic[0]	Plastic[0]	Plastic[0]	Stainless steel[0]	Plastic[0]	Plastic[0]
Nipple drinker	Stainless steel[0]	Stainless steel[0]	Stainless steel[0]	Stainless steel[0]	Stainless steel[0]	Stainless steel[0]
Nipple drinker	Stainless steel[0]	Stainless steel[0]	Stainless steel[0]	Stainless steel[0]	Stainless steel[0]	Stainless steel[0]
Trough, outside	Stainless steel[0]	Stainless steel[0]	Stainless steel[0]	Plastic[0]	Stainless steel[0]	Stainless steel[0]
Trough, inside	Stainless steel[0]	Stainless steel[0]	*	Stainless steel[0]	Stainless steel[0]	*
Manipulable material 1	Plastic + Metal[0]	*	Plastic[0]	Stainless steel[0]	Plastic + Metal[0]	Plastic[0]
Manipulable material 2	Stainless steel[0]	*	Wood[1]	Wood[1]	Stainless steel[0]	Plastic + Metal[0]
Window sill	Concrete[1]	*	*	Concrete[1]	Plastic[0]	Concrete[1]
Feeding tube inside	Plastic[0]	Plastic[0]	Plastic[0]	*	*	*

*Sampling not possible.
[0]Smooth surface
[1]Porous surface

Supplemental table 6.2. The different displayed techniques were used to evaluate the methods, reveal critical points in hygiene or to assess the effect of hygiene training.

		Method evaluation		Hygienic critical points		Training effect	
	Sampling time	1st	2nd	1st	2nd	1st	2nd
Microbiological swabs	TVC[1]	x	x	x	x	x	x
	TCC[2]	x	x				
	MRSA[3]			x	x	x	x
	ESBL[4]						x
Rapid test swabs	ATP[5]	x	x	x	x	x	x
	Protein	x	x	x	x	x	x
Agar contact plates	TVC	x					
	Enterobacteriaceae with VRBD[6]	x					
	TVC with disinfectant neutralizer with DE[7]	x					
Other	Water samples			x	x	x	x
	Sock samples			x	x	x	x

[1]Aerobic total viable count
[2]Total coliform count
[3]Methicillin-resistant *Staphylococcus aureus*
[4]Extended-spectrum β-lactamase-producing bacteria
[5]Adenosine triphosphate
[6]Violet red bile dextrose agar
[7]Dey Engley Agar

6.2. Supplemental information to Chapter 3

Interview Sheet (filled out by the sampler with answers from the respondent for the farm)

Date:

Address:

Name of the respondent:

1. How old are you?
2. Which professional qualification do you have?
   □ Journeyman □ Advanced journeyman □ Foremen
   □ Bachelor □ Master
3. Have you ever taken further training courses in the field of animal welfare, handling of animals or hygiene?
   □ Yes □ No
   If yes, please name title, presenter, place and date of the course:
4. How many dairy cows in total are kept on this farm?
5. What is the breed of your calves?
6. How many cows are born each year?
7. What is the replacement rate?
8. How many persons are employed on the farm that have regular contact with the cows and calves?
9. Who else has contact with the cows and calves?
10. Who takes care of the calves?
    □ Farm manager □ Employee □ Wife or mother of the farm manager
    □ Temporary help □ Trainees □ Other:
11. Is there a regular treatment by a veterinarian for the calves (e.g., vaccination, antibiotic treatment)? □ Yes □ No
    If so, please specify active agent, frequency and date:
12. Is there a regular consultation by a veterinarian for the calves? □ Yes □ No
    If so, what does it look like?
13. Do you regularly consult other advisors for calf housing? □ Yes □ No
    If so, in which subject?
14. How many places do you have for calves?
15. How many pens / huts for individual housing of calves do you have?
16. How does the parturition mainly proceed?
    □ Rare complications □ Frequent complications
17. Do you use a mechanical calf puller? □ Yes □ No
    If so, how often do you use it? □ Rarely □ Occasionally □ Frequently
18. How often does dystocia occur?
19. How do you record data about the occurrence of dystocia?
    □ Not recorded □ Estimated □ Documentation of each case

20. How often are the data of the occurrence of dystocia analyzed?
□ Weekly □ Monthly □ Every six months □ Once a year □ Other:
21. What is the actual number of calf losses during the first 14 days of life?
22. When do calf losses most likely occur?
□ First week □ Second week □ Other:
23. How often is the number of calf losses during the first 14 days assessed?
□ Weekly □ Monthly □ Every six months □ Once a year □ Other:
24. What is the actual mortality rate for suckling calves until weaning?
25. How long are calves kept in individual housing?
26. What kind of individual housing do you use?
□ Adjacent pens in a stable □ Adjacent huts outside
□ Isolated pens in a stable □ Isolated huts outside □ Other:
27. If calf huts are present, are they moved after each calf before restocking?
□ Yes □ No
If so, how far is the distance between locations [m]?

Please, describe the moving concept:

28. If calf huts are present, are the huts stored in stacks when not in use?
□ Yes □ No
29. How often is new bedding material provided?
□ Daily □ Once per week □ After each calf
30. How are the stables for individual housing occupied?
□ Continuously □ In groups □ All in all out
31. How are the calving pens occupied?
□ Continuously □ In groups □ All in all out
32. How long is the minimum time the pens / huts for single housing remain empty [d]?
33. How long is the minimum time calving pens remain empty [d]?
34. How often are the calves rehoused [d]?
35. How are the calves rehoused? □ Moved by feet □ Calf taxi
□ Carried □ Lead with a halter □ Other:
36. After how many days are the calves transferred to group-housing [d]?
37. After how many hours are newborn calves separated from the dam?
□ Always immediately □ < 4 hours □ 5-8 hours □ 24 hours
□ more than 24 hours. If so, please state the exact time:
38. Is it ensured that newborn calves are fed with colostrum?
□ Yes □ No
If yes, please describe how you ensure that:

39. How is the colostrum fed? □ Suckling the dam □ Feeding bottle
□ Drenching □ Feeding bucket with artificial teat □ Other
40. How much colostrum is fed?
Please indicate the minimum and maximum value.

41. Are colostrum reserves stored? □ Yes □ No
If yes, please describe.

42. Is the colostrum of primiparous cows fed? □ Yes □ No

43. Is the quality of the colostrum routinely checked?
□ Yes □ No
If yes, please describe:

44. How (technically) are the calves fed in individual housing?
□ Feeding bucket with artificial teats □ Automatic milk feeder
□ Trough or bucket □ Suckling the dam □ Other:

45. What is used for feeding calves in individual housing?
□ Milk replacer □ Whole milk □ Waste milk
□ Milk with high somatic cell count □ Other

46. How was the milk feeding prepared?
□ Warm □ Cold □ Acidified □ Other supplements:

47. How often are the calves fed?
□ Once a day □ Twice a day □ Other:

48. Are there any differences in feeding male and female calves? □ Yes □ No
If yes, what are the differences?

49. After how many weeks are the calves weaned [w]?

50. How often is drinking water changed [h]?

51. At what age is roughage fed to the calves [d]?

52. At what age is concentrate fed to the calves [d]?

53. Are the calves treated with deworming agents? □ Yes □ No
If yes, how often are the calves dewormed [w]?

54. How often does diarrhea occur in the calves?
□ 0% □ < 5% □ > 5%

55. Have fecal analyses been carried out in calves with diarrhea? □ Yes □ No
If yes, what was the result?

56. Were the calves analyzed for Salmonella during the last three years?
□ Yes □ No

57. If so, where was Salmonella detected, and which measures were taken?
□ Yes □ No □ Measures taken:

58. Were the calves analyzed for Cryptosporidia during the last three years?
□ Yes □ No

59. If so, where was Cryptosporidia detected, and which measures were taken?
□ Yes □ No □ Measures taken:

60. Are there any other frequently occurring health disorders in individually housed calves?
□ Yes □ No
If so, which?

61. Are the calves treated with veterinary medicinal products during the last 6 months?
□ Yes □ No
If so, with which?

62. Have feed additives been supplemented to the calves during the last 6 months?
□ Yes □ No
If so, which?

63. Who is responsible for sanitation?

	Calving pen	Individual housing equipment
Farm manager	□	□
Employees	□	□
Temporary help	□	□
Trainees	□	□
Other:	□	□

64. How often is cleaning and disinfection carried out [d]?

	Cleaning	Disinfection
Calving pens		
Single house pens/huts		
Feeding buckets		
Hay racks		
Water troughs		

65. What chemical agents are used for cleaning and disinfection?

	Cleaning	Disinfection
Calving pens		
Single house pens/huts		
Feeding buckets		
Hay racks		
Water troughs		

66. Are the feeding buckets disassembled for each cleaning? □ Yes □ No

67. How long is the interval between cleaning and disinfection measures and restocking [d]?

Record Sheet (filled out by sampler)

Date:

Address:

Name of the contact person:

Access to barn

1. Are changing rooms available? □ Yes □ No
2. Are hand washing basins available? □ Yes □ No
3. Are hand disinfectants available? □ Yes □ No
   Notes:

Overall impression

4. Housing category: □ Pens □ Huts
5. Climate: □ Inside □ Outside
6. Lighting conditions [lx]:
   Notes:

Characterization of pens / huts:

7. Do the pens / huts have an additional enclosure? □ Yes □ No
8. Is there a slope of the floor and in which direction?
   □ Back □ Front □ Side
9. How much distance is between the calf pens / huts [m]?
10. Are individual pens / huts movable? □ Yes □ No
11. What size are the gaps between grids or fencing [m]?
12. Is a direct contact between the calves possible? □ Yes □ No
13. How are the pens / huts arranged?
    □ Side by side □ In a row □ Other:
14. What material is the floor made of?
    □ Concrete plate □ Concrete paving □ Sand □ Grass block pavers
    □ Clay/earth □ Wood □ Other:
15. Is there any unevenness, such as scratches or cracks? □ Yes □ No
    If yes, please take pictures!

    Maximal depth of scratches and cracks [mm]?
16. What material is the grid / fencing made of? □ Plastic □ Metal □ Other:
17. What material are the pens / huts made of? □ Plastic □ Metal □ Wood
    □ Concrete □ Other:
18. How far is the nearest water supply [m]?
19. Are the calves drenched? □ Yes □ No
    If yes, where and how is the drenching tube stored?

Visual assessment of the sampling points

①= no remaining soiling visible

② = minor soiling visible

③= coarse soiling visible

Please, mark the most probable number. Please cross out terms if sampling is not possible and state why.

Pen / Hut 1

Front wall /grid	①	②	③
Back wall	①	②	③
Side wall left	①	②	③
Floor	①	②	③
Hay rack	①	②	③
Ceiling	①	②	③
Fixture for feeding buckets	①	②	③

Pen / Hut 2

Front wall /grid	①	②	③
Back wall	①	②	③
Side wall left	①	②	③
Floor	①	②	③
Hay rack	①	②	③
Ceiling	①	②	③
Fixture for feeding buckets	①	②	③

Feeding bucket 1

Feeding bucket, outside	①	②	③
Feeding bucket, inside	①	②	③
Artificial teat, outside	①	②	③

Artificial teat, inside	①	②	③

Feeding bucket 2

Feeding bucket, outside	①	②	③
Feeding bucket, inside	①	②	③
Artificial teat, outside	①	②	③
Artificial teat, inside	①	②	③

Drawing of the pen / huts with sample points marked:

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